524 Rec'd PCT/PTO 29 OCT 1999

FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1721-18
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5) 09/403980 Unknown
INTERNATIONAL APPLICATION NO. PCT/FR98/00883	INTERNATIONAL FILING DATE April 30, 1998	PRIORITY DATE CLAIMED 30 April 1997 28 January 1998

TITLE OF INVENTION
POLYPEPTIDES ASSOCIATED WITH ACTIVATOR RECEPTORS AND THEIR BIOLOGICAL APPLICATIONS

APPLICANT(S) FOR DO/EO/US
VIVIER et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☒ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)) **including translation of amended sheets of claims. Paper and computer readable translation of Sequence Listing.**

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment **including New Figure 16 and highlighted copies of Figures 16, 10, 12, 14, 13, 11 and 15.**
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information. Copy of Request, PTO-1449 and International Search Report,

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/403980 Unknown	PCT/FR98/00883	1721-18

17. ☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):

-- Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....$970.00

-- International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00

-- International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO.....$760.00

-- International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00

-- International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	840.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).						$	130.00
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE			
Total Claims	**25**	-20 =	5	X	$18.00	$	90.00
Independent Claims	**2**	-3 =	0	X	$78.00		0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				+$260.00		$	0.00
TOTAL OF ABOVE CALCULATIONS =						$	**1060.00**
Reduction by ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).							0.00
SUBTOTAL =						$	**1060.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +							0.00
TOTAL NATIONAL FEE =						$	**1060.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +						$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1,210 – Small Entity Fee = $605)						$	0.00
TOTAL FEES ENCLOSED =						$	**1060.00**
						Amount to be: refunded	$
						charged	$

a. ☒ A check in the amount of $1060.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201
Telephone: (703) 816-4000

SIGNATURE

B.J. Sadoff
NAME

36,663 **October 29, 1999**
REGISTRATION NUMBER Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

VIVIER et al

Atty. Ref.: 1721-18

U.S. National Phase of PCT/FR98/00883
Serial No. Unknown

Group: Unassigned

Filed: October 29, 1999

Examiner: Unassigned

For: POLYPEPTIDES ASSOCIATED WITH ACTIVATOR RECEPTORS AND THEIR BIOLOGICAL APPLICATIONS

* * * * * * * * * * * *

October 29, 1999

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Preliminarily amend the attached English translation of the above-identified application which includes an English translation of originally presented claims 1-31 and an English translation of claims 1-25 which were presented in an amendment pursuant to Article 34 during the international phase.

IN THE SPECIFICATION:

Please amend the specification as follows.

Insert the attached Sequence Listing before the Figures and renumber any subsequent pages as may be required.

IN THE ABSTRACT:

Add the attached Abstract after the claims.

IN THE FIGURES:

Insert the attached new Figure 16.

IN THE CLAIMS:

Amend the claims 1-25 noted on pages 36-40 of the application as "MODIFIED PAGE" which were submitted as an amendment under Article 34 and are presently pending.

Claim 3, line 1, delete "or 2".

Claims 4-10, line 1, delete "any one of the preceding claims" and insert --claim 1--.

Claim 13, line 3, delete "any one of the preceding claims" and insert --claim 1--.

Claim 14, line 1, delete "17" and insert --13--.

Claim 15, line 4, delete "any one of the preceding claims" and insert --claim 1--.

Claim 17, lines 1-2, delete "any one of the preceding claims" and insert --claim 11--.

Claim 19, lines 1-2, delete "any one of claims 1 to 12" and insert --claim 1--.

Claim 20, line 2, delete "any one of claims 1 to 12" and insert --claim 1--; and

lines 11-12, delete "a polypeptide according to any one of claims 1 to 12" and insert --said polypeptide--.

21. (Amended) Pharmaceutical composition comprising, in association with a pharmaceutically acceptable vehicle, an effective amount of polypeptides according to claim 1 [any one of the preceding claims], or fragments of such polypeptides, or an effective amount of antibodies specific thereto [according to claim 13 or 14], or fragments of such antibodies, or an effective amount of nucleic acids encoding said polypeptides or fragments of said polypeptides [according to claim 15 or 16], or variants of such nucleic acids.

22. (Amended) *In vitro* method of diagnosing an abnormal or undesired function of a cell, characterized in that it comprises steps involving:

- bringing of at least one cell, or one cell extract, into contact with an antibody according to claim 13 [or 14], or a fragment of such an antibody, or with a nucleic acid which encodes a polypeptide which specifically binds to said antibody [according to claim 15 or 16], or a variant of such a nucleic acid, and

- revealing of the reaction product which may be formed.

23. (Amended) *In vitro* diagnostic method according to claim 22, characterized in that said abnormal or undesired function results in an immunoproliferative disease, an immunodeficiency disease such as an HIV disease, a cancer such as lymphoproliferative disease of the granular lymphocytes, an autoimmune disease such as rheumatoid arthritis, an infectious disease such as malaria, an allergic response or a graft reject.

24. (Amended) Method of identifying molecules which adaptor carryout the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with polypeptides according to [any one of claims 1 to 12] claim 1 (or with fragments of such polypeptides), and

ii. selection of those candidate molecules for which a binding to said polypeptides (or to said polypeptide fragments) is observed.

25. (Amended) Method of identifying molecules capable of modulating a cell activity resulting from the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with molecules which adapt or carry out the activation of a KAR, as obtained by the method according to claim 24, [and with polypeptides according to any one of claims 1 to 12 (or with fragments of such polypeptides)], and

ii. selection of those candidate molecules which exert an effect on the binding between said polypeptides (or said polypeptide fragments) and said adapter of effector molecules, as observed in the absence of said candidate molecules.

REMARKS

Favorable consideration of the attached and entry of the above amendments are requested.

The specification has been amended to insert the attached Sequence Listing. A paper and computer readable copy of the Sequence Listing are attached. The attached paper and computer copies of the Sequence Listing are the same. No new matter has been added.

The specification has been added to include, on a separate page, the Abstract of Application No. PCT/FR98/00883, which is the present case.

The claims have been amended to be in more traditional U.S. formal.

The originally-filed Figure 16, has been replaced by the attached amended Figure 16, which includes the missing information of the originally-filed Figure 16, as supported by the specification. No new matter has been added. Support for the change is detailed below.

Figure 16 has been amended in the "consensus" sequence, as the last two amino acids of the three sets of 10 amino acids at the right margin of the page were not included in the originally-filed Figure 16. The applicants note, however, that one of ordinary skill in the art would appreciate that the representation of sequences in Figure 16 should have included sets of 10 amino acids and the three sets on the right margin only contain eight (see, circled and highlighted sets of amino acids). As noted in the originally-filed Figure 16, this figure shows the alignment of SEQ ID NOs: 11, 13, 15, 14 and 12 to form the consensus SEQ ID NO: 17.

SEQ ID NOs: 11, 13, 15, 14 and 12, as originally-filed are shown in Figures 10B, 12B, 14B, 13B and 11B (copies attached with missing amino acids of original Figure 16 highlighted), respectively. One of ordinary skill would appreciate that the missing amino acids of Figure 16 are "TF" in the first set (i.e., amino acids 49 and 50 of the consensus sequence, amino acids 49 and 50 of Figure 10B, (SEQ ID NO: 11), amino acids 22 and 23 of Figure 12B (SEQ ID NO: 13), amino acids 20 and 21 of Figure 14B (SEQ ID NO: 15), amino acids 44 and 45 of Figure 13B (SEQ ID NO: 14), and amino acids 36 and 37 of Figure 11B (SEQ ID NO: 12)). Similarly, one or ordinary skill would appreciate that the missing amino acids of the second set in Figure 16 are "RK" (amino acids 99 and 100 of the consensus sequence). See, amino acids 99 and 100 of SEQ

ID NO: 11 (Figure 10B), amino acids 71 and 72 of SEQ ID NO: 13 (Figure 12B), amino acids 70 and 71 of SEQ ID NO: 15 (Figure 14B), amino acids 94 and 95 of SEQ ID NO: 14 (Figure 13B) and amino acids 86 and 87 of SEQ ID NO: 12 (Figure 11B). Finally, one of ordinary skill would appreciate the amino acids missing from the third block of ten amino acids in the originally-filed Figure 16 are "SR" (amino acids 149 and 150 of the consensus sequence). See amino acids 149 and 150 of SEQ ID NO: 11 (Figure 10B), and amino acids 122 and 123 of SEQ ID NO: 13 (Figure 12B).

Moreover, the corresponding DNA consensus sequence shown in the attached copy of Figure 15 wherein the correct DNA encoding the amino acid consensus sequence of originally-filed Figure 16 is circled and highlighted in pink, further demonstrates the applicants were in possession of the correct sequence and inserting of the attached amended Figure 16 does not enter new matter. A separate Request to the Chief Draftsperson for approval to amend Figure 16 as noted, is attached.

Entry of the above and attached, and an early Action of the merits are requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B.J. Sadoff
Reg. No. 36,663

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ABSTRACT

The invention concerns novel means for diagnosing, preventing, compensating, treating an abnormal or unwanted functioning of KAR receptors (*Killer cell Activatory Receptor*), counterparts of non-inhibiting KIR receptors (*Keller cell Inhibitory Receptors*) of the immunoglobulin or lectin type. The invention concerns in particular, novel KARAP (*KAR-Associated Proteins*) polypeptides and their biological applications. A KARAP polypeptide is naturally associated with a KAR receptor, and in the absence of such a KARAP, said KAR receptor is naturally incapable of transducing an activating signal that can be detected. The application also concerns methods for obtaining or identifying such KARAP polypeptides.

Figure 16

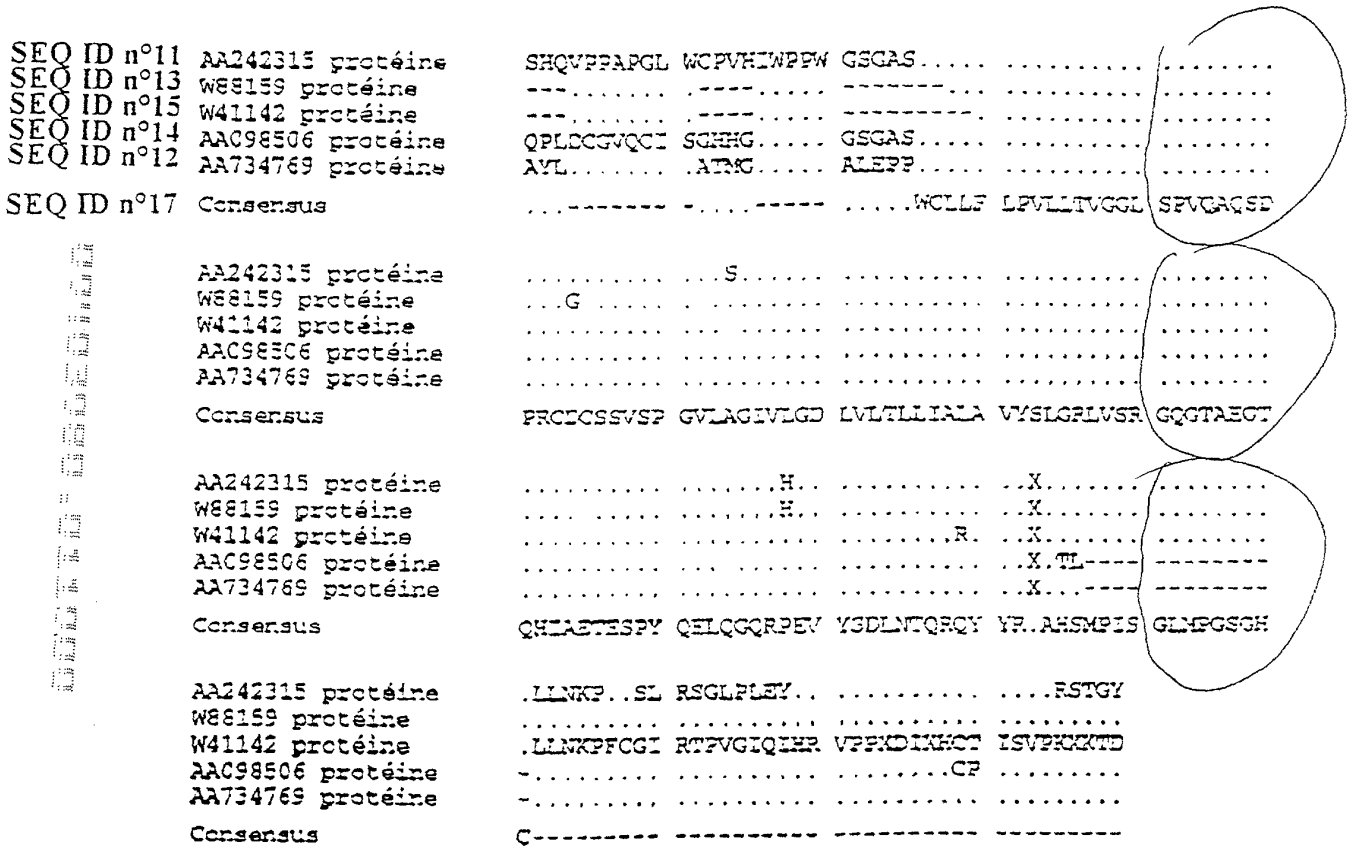
SEQ ID n°11 AA242315 protéine SHQVPPAPGL WCPVHIWPPW GSGAS
SEQ ID n°13 W88159 protéine
SEQ ID n°15 W41142 protéine
SEQ ID n°14 AAC98506 protéine QPLDCGVQCI SGHHG GSGAS
SEQ ID n°12 AA734769 protéine AYL ATMG ALEPP
SEQ ID n°17 Consensus .....WCLLF LPVLLIVGGL SPVGAQSD
AA242315 protéine ...S
W88159 protéine ...G
W41142 protéine
AAC98506 protéine
AA734769 protéine
Consensus PRCICSSVSP GVLAGIVLGD LVLTLLIALA VYSLGPLVSR GQGTAECT
AA242315 protéine .....H..
W88159 protéine .....H..
W41142 protéine .....R.
AAC98506 protéine
AA734769 protéine
Consensus QHIAETESPY QELQGQRPEV YSDLNTQRQY YR..AHSMPIS GLMPGSGH
AA242315 protéine .LLNKP..SL RSGLPLEY.. ....RSTGY
W88159 protéine
W41142 protéine .LLNKPFCGI RTPVGIQIHR VPPKDIKHCT ISVPKKKTD
AAC98506 protéine .....CP
AA734769 protéine
Consensus C

Figure 10A

SEQ ID n°6

```
                                                  10        20        30        40        50
          1234567890 1234567890 1234567890 1234567890 1234567890
TCACACCAGG TCCCACCAGC CCCTGGACTG TGGTGTCCAG TGCATATCTG     50
GCCACCATGG GGCTCTGGAG CCTCCTGGTG CCTTCTGTTC CTTCCTGTCC     100
TCCTGACTGT GGCAGGATTA AGTCCCGTAC AGGCCCAGAG TGACACTTTG     150
CCAAGATGCG ACTGTTCTTC CGTGAGCCCT GGTGTACTGT CTGGGATTGT     200
TCTGGGTGAC TTGGTGTTGA CTCTGCTGAT TGCCCTGGCT GTGTACTCTC     250
TGGGCCGCCT GGTCTCCCGA GGTCAAGGGA CAGCGGAGG GACCCGGAAA     300
CAACACATTG CTGAGACTGA GTCGCCTTAT CAGGAGCTTC AGGGTCAGAC     350
ACATGAAGTA TACAGTGACC TCAACACACA GAGGCAATAC TACAGATGAG     400
CCCACTCTAT GCCCATCAGC GGCCTGATGC CCGGATCCGG TCATTCCAGN     450
TGCCTACTCA ACAAGCCCTC TCTGAGTCA GGACTCCCGT TGCAATACAG     500
ATCCACAGGG TACCT                                          515
```

```
                                                  10        20        30        40        50
          1234567890 1234567890 1234567890 1234567890 1234567890
SHQVPPAPGL WCPVHIWPPW GSGASWCLLF LPVLLTVGGL SPVQAQSDTF     50
PRCDCSSVSP GVLSGIVLGD LVLTLLIALA VYSLGRLVSR GQGTAEGTRK     100
QHIAELESPY QELQGQRHEV YSDLNTQRQY YRKAHSMPIS GLMPGSGHSR     150
CLLNKPSLRS GLPLEYRSIG Y                                   171
```

Figure 10B

SEQ ID n°11

Figure 12A

SEQ ID n°12

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
GCCTTCTGTT CCTTCCTGTC CTCCTGACTG TGGCAGGATT AAGTCCCGTA     50
CAGGCCCAGA GTGACACTTT CCCAAGATGC GGCTGTTCTT CCGTGAGCCC    100
TGGTGTACTG GCTGGGATTG TTCTGGGTGA CTTGGTGTTG ACTCTGCTGA    150
TTGCCCTGGC TGTGTACTCT CTGGGCCGCC TGGTCTCCCG AGGTCAAGGG    200
ACAGCCGAAG GGACCCGGAA ACAACACATT GCTGAGACTG AGTCGCCTTA    250
TCAGGAGCTT CAGGGTCAGA GACATGAAGT ATACAGTGAC CTCAACACAC    300
AGAGGCAATA TTACAGATGA GCCCACTCTA TGCCCATCAG CGGCCTGATG    350
CCCGGATCCG GTCATTCCAG ATGCCT                              376
```

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
LLFLPVLLTV GGLSPVQAQS DTFPRCGCSS VSFGVLAGIV LGDLVLTLLI     50
ALAVYSLGRL VSRGQGTAEG TPKQHIAETE SPYQELQGQR HEVYSDLNTQ    100
RQYYRKAHSM PISELMFGSG HSFC                                124
```

Figure 12B

SEQ ID n°13

Figure 14A

SEQ ID n°10

```
          10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
GTTCCTTCCT GTCCTCCTGA CTGTGGCAGG ATTAAGTCCC GTACAGGCCC    50
ACAGTGACAG TTTGCCAAGA TGCGACTGTT CTTCCGTGAG CCCTGGTGTA   100
CTGGCTGGGA TTGTTCTGGG TGACTTGGTG TTGACTCTGC TGATTGCCCT   150
GGCTGTGTAC TCTCTGGCCC GCCTGGTCTC CCGAGGTCAA GCGACAGCGG   200
AAGGGACCCG GAAACAACAC ATTGCTGAGA CTGAGTCGCC TTATCAGGAG   250
CTTCAGGGTC AGAGACCTGA AGTATACAGT GACCTCAACA CACAGACGCG   300
ATATTACAGA TGAGCCCACT CTATGCCCAT CAGCGGCCTG ATGCCCCGAT   350
CCGGTCATTC CAGATGCCTA CTCAACAAGC CCTTCTGTCG GATCAGGACT   400
CCCGTTGGAA TACAGATCCA CAGGGTACCT CCCTGAGATA TCTGACATTG   450
TACCATTTCT GTCCCCAAAT AGAAGACGCA CA                      482
```

```
          10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
FLPVLLTVGG LSPVQAQSDT FFRCDCSSVS PGVLAGIVLG DLVLTLLIAL    50
AVYSLGRLVS RGQGTAEGTR KQHIAETESP YQELQGQRPE VYSDLNTQRR   100
YYRKAHSMPI SGLMPGSGHS RCLINKPFCG IRTPVGIQLH RVPPKDIKHC   150
TISVPKKATD                                               160
```

Figure 14B

SEQ ID n°15

Figure 13A

SEQ ID n°9

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
CCAGCCCCTG GACTGTGGTG TCCAGTGCAT ATCTGGCCAC CATGGGGGCT     50
CTGGAGCCTC CTGGTGCCTT CTGTTCCTTC CTGTCCTCCT GACTGTGGGA    100
GGATTAAGTC CCGTACAGGC CCAGAGTGAC ACTTTGCCAA GATGCGACTG    150
TTCTTCCGTG AGCCCTGGTG TACTGGCTGG GATGTTCTG GGTGACTTGG    200
TGTTGACTCT GCTGATTGCC CTGGCTGTGT ACTCTCTGGG CCGCCTGGTC    250
TCCCGAGGTC AAGGGACAGC CGAACGGACC CGGAAACAAC ACATTGCTGA    300
GACTGAGTCG CCTTATCAGG AGCTTCAGGG TCAGAGACCA GAAGTATACA    350
GTGACCTCAA CACACAGAGG CAATATTACA GATGAGCCAC TCTATGCCCA    400
TC                                                       402
```

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
QPLDCGWQCI SGHRGSGAS WCLLFLPVLL TVGGLSPVQA QSDIFPRCDC     50
SSVSPGVLAG IVLGDLVLTL LIALAVYSLG RLVSPGQGTA EGTRKQHTAE    100
TESPYQELQG QRPEVYSDLN TQRQYYRKAT LCP                      133
```

Figure 13B

SEQ ID n°14

Figure 11A

SEQ ID n°7

```
         10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
GTGCATATCT GGCCACCATG GGGGCTCTGG AGCCTCCATG GTGCCTTCTG     50
TTCCTTCCTG TCCTCCTGAC TGTGGGAGGA TTAAGTCCCG TACAGCCCCA    100
GAGTGACACT TTGCCAAGAT GCGACTGTTC TTCCGTGAGC CCTGGTGTAC    150
TGGCTGGGAT TGTTCTGGGT GACTTGGTGT TGACTCTGCT GATTGCCCTG    200
GCTGTGTACT CTCTCGGCCG CCTGGTCTCC CCAGGTCAAG GGACAGCGGA    250
AGGGACCCGG AAACAACACA TTGCTGAGAC TGAGTCGCCT TATCAGGAGC    300
TTCAGGGTCA GGAGCCAGAA GTATACAGTG ACCTCAACAC ACAGAGGCAA    350
TATTACAGAT GAGCCCACTC T                                   371
```

```
         10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AYLATMGALE PFWCLLFLPV LITVGGLSFV QAQSDTFPRC DCSSVSPGVL     50
AGIVLGELVL TLLIALAVYS LGRLVSRGQG TAEGIRKQHI AETESPYQEL    100
QGQRPEVYSD LNTQRQYYRX AHS                                 123
```

Figure 11B

SEQ ID n°12

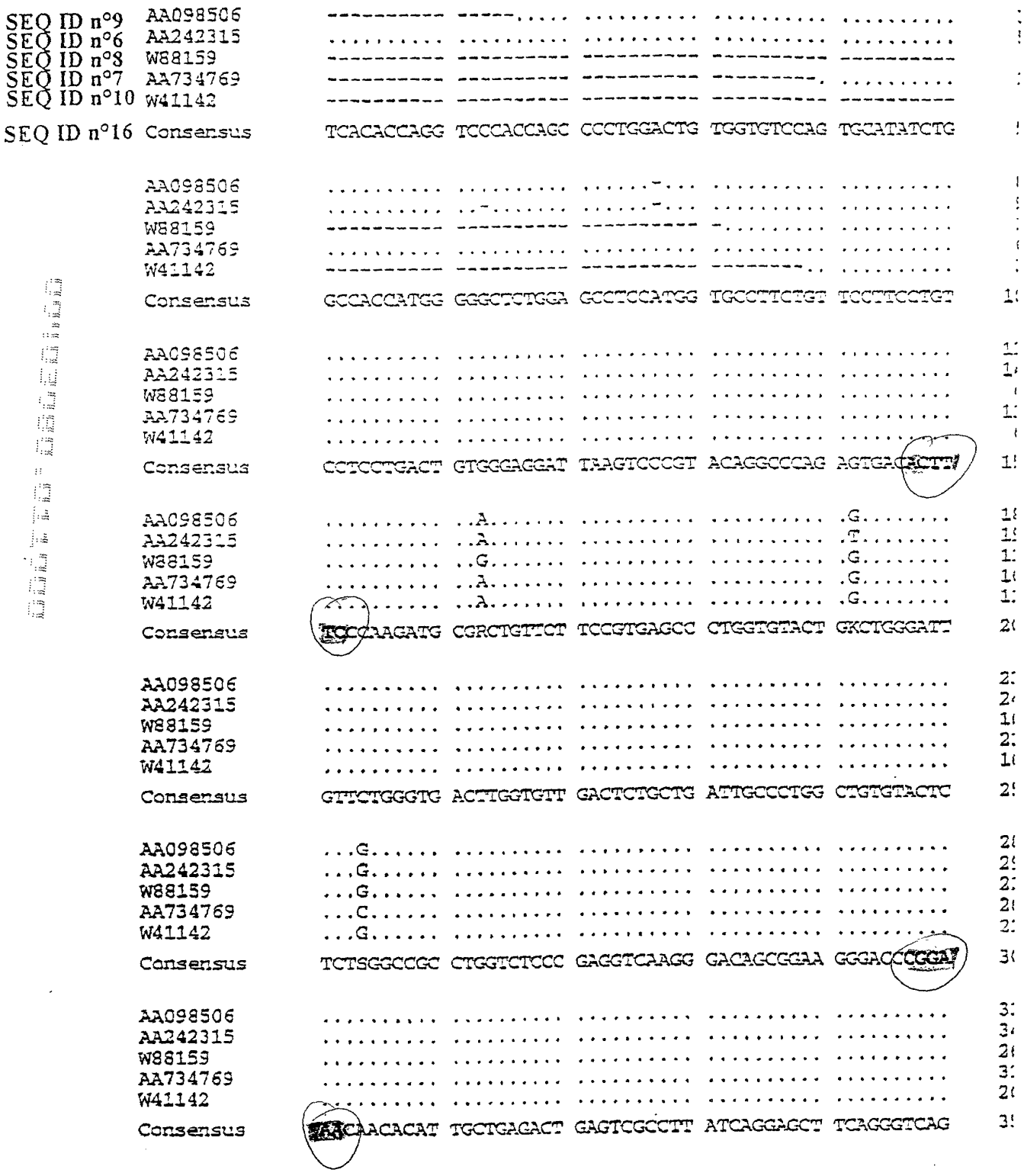
SEQ ID n°9 AA098506
SEQ ID n°6 AA242315
SEQ ID n°8 W88159
SEQ ID n°7 AA734769
SEQ ID n°10 W41142
SEQ ID n°16 Consensus
TCACACCAGG TCCCACCAGC CCCTGGACTG TGGTGTCCAG TGCATATCTG
AA098506
AA242315
W88159
AA734769
W41142
Consensus
GCCACCATGG GGGCTCTGGA GCCTCCATGG TGCCTTCTGT TCCTTCCTGT
AA098506
AA242315
W88159
AA734769
W41142
Consensus
CCTCCTGACT GTGGGAGGAT TAAGTCCCGT ACAGGCCCAG AGTGACACTT
AA098506
AA242315
W88159
AA734769
W41142
Consensus
TCCCAAGATG CGRCTGTTCT TCCGTGAGCC CTGGTGTACT GKCTGGGATT
AA098506
AA242315
W88159
AA734769
W41142
Consensus
GTTCTGGGTG ACTTGGTGTT GACTCTGCTG ATTGCCCTGG CTGTGTACTC
AA098506
AA242315
W88159
AA734769
W41142
Consensus
TCTSGGCCGC CTGGTCTCCC GAGGTCAAGG GACAGCGGAA GGGACCCGGA
AA098506
AA242315
W88159
AA734769
W41142
Consensus
AACAACACAT TGCTGAGACT GAGTCGCCTT ATCAGGAGCT TCAGGGTCAG

Figure 15 (suite)


```
AA098506    ....CA.... .......... .......... .......A.. ..........  3(
AA242315    ....AT.... .......... .......... .......A.. ..........  3(
W88159      ....AT.... .......... .......... .......A.. ..........  3(
AA734769    ....CA.... .......... .......... .......A.. ..........  3(
W41142      ....CT.... .......... .......... .......G.. ..........  3(
Consensus   AGACMWGAAG TATACAGTGA CCTCAACACA CAGAGGCRAT ATTACAGATG  4(

AA098506    ....-..... ..........- ---------- ---------- ----------  4(
AA242315    .......... .......... .......... .......... ..........  4(
W88159      .......... .......... .......... .......... ..........  3(
AA734769    .......... ---------- ---------- ---------- ----------  3(
W41142      .......... .......... .......... .......... ..........  3(
Consensus   AGCCCACTCT ATGCCCATCA GCGGCCTGAT GCCCGGATCC GGTCATTCCA  4(

AA098506    ---------- ---------- ---------- ---------- ----------  4(
AA242315    .......... .......... .-..C..A.. .......... ..........  4(
W88159      .......--- ---------- ---------- ---------- ----------  3(
AA734769    ---------- ---------- ---------- ---------- ----------  3(
W41142      .......... .......... ....G..G.. .......... ..........  4(
Consensus   CRTGCCTACT CAACAAGCCC TTCTSTGRGA TCAGGACTCC CGTTGGAATA  5(

AA098506    ---------- ---------- ---------- ---------- ----------  4(
AA242315    .......... .........-- ---------- ---------- ----------  5(
W88159      ---------- ---------- ---------- ---------- ----------  3(
AA734769    ---------- ---------- ---------- ---------- ----------  3(
W41142      .......... .......... .......... .......... ..........  4(
Consensus   CAGATCCACA GGGTACCTCC CTGAGATATC TGACATTGTA CCATTTCTGT  5(

AA098506    ---------- ----------                                    4(
AA242315    ---------- ----------                                    5(
W88159      ---------- ----------                                    3(
AA734769    ---------- ----------                                    3(
W41142      .......... ..........                                    4(
Consensus   CCCCAAATAG AAGACGGACA                                    5(
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NEW POLYPEPTIDES ASSOCIATED WITH ACTIVATORY RECEPTORS AND THEIR BIOLOGICAL APPLICATIONS

The invention relates to new particular polypeptides capable of transducing a signal originating from an activatory receptor for class I MHC molecules, functioning both as an autonomous receptor or as a co-receptor, and a KAR (*Killer-cell Activatory Receptor*), in particular to the antibodies obtained from said polypeptides serving as immunogens, and to the nucleic acids corresponding to said polypeptides.

The invention also relates to the processes for obtaining such polypeptides and to the biological, more particularly, preventive, therapeutic and diagnostic applications, of said polypeptides, antibodies and nucleic acids.

In order to maintain the coherence and to ensure the integrity of the body, the immune system must bring into play a coordinated system of intercellular communications.

Different types of receptors are involved in these communications.
Three of them, namely the receptors for the antigen of B lymphocytes (BCR), the receptors for the antigen of T lymphocytes (TCR) and the receptors recognizing the Fc portion of antibodies (RFc), are now well described and their different structures are relatively well known.

Other receptors which are neither receptors for antigens, nor receptors for antibodies have been described but their structures and action mechanisms are still little known.

These are the receptors for molecules of MHC (Major Histocompatibility Complex) such as KARs (*Killer cell Activatory Receptors*) and their inhibitory counterparts, KIRs (*Killer cell Inhibitory Receptors*).

KARs and KIRs are not limited to NK cells: they are also naturally expressed by T cells.

KARs are highly homologous with KIRs (up to 96% homology between KARs and KIRs at the extracytoplasmic level).

However, KARs and KIRs do not perform the same functions: KIRs are involved in the negative (inhibitory) control of the activation of NK and T cells, whilst KARs are involved in the positive (stimulatory) control of the activation of NK and T cells.

Major differences as regards the trans- and intracytoplasmic domains were demonstrated between the activatory isoform (KAR) and inhibitory isoform (KIR).

In fact, unlike KIRs, KARs express a charged amino acid residue (lysine) in their transmembrane domain and do not contain an ITIM unit (immunoreceptor

inhibition unit based on tyrosine residue(s)) in their intracytoplasmic domain. For all that the monomeric KAR receptors do not contain an ITAM unit (immunoreceptor activation unit based on tyrosine residue(s)).

The situation observed for KARs, activatory receptors for MHC molecules, and members of the IgSF (immunoglobulins superfamily), namely an activatory receptor, counterpart of an ITIM inhibitory receptor, itself presenting neither ITIM, nor ITAM but presenting a transmembrane charged amino acid (lysine, arginine, aspartic acid, glutamic acid), can be observed for other types of receptors. This is so for the case of activatory (or at the very least non inhibitory) receptors for MHC molecules, such as NKG2C/D (which is of lectin type and the inhibitory counterpart of which is NKG2A/B), but also for other non inhibitory receptors, such as SIRP β and ILT 1, the ligands of which are still unknown and which have been described either as hematopoietic cells and on non-hematopoietic cells (SIRP β), or on B cells, macrophages and dendritic cells (ILT1).

KARs can function as autonomous receptors, in particular for class I MHC molecules. Thus it is known that the engagement of KARs with class I MHC molecules expressed on the surface of target cells, initiates the lymphocyte activation programmes as established by the fact of Ca^{2+} intracytoplasmic mobilisation and the induction of lysis of the target cells.

In addition to their functions as autonomous receptors for MHC molecules, KARs can also perform co-receptor functions for TCR and RFc receptors (Mandelboim O. *et al.*, 1996, *Science 274*:2097; Cambiaggi A. *et al.*, 1996, *Blood 87*:2369).

In fact, during the recognition of constant fragments (cF) of immunoglobulins G (IgG) by receptors such as CD16 (RFcγIII), and during the recognition of antigens by the CD3/TCR complex restricted by class I or II MHC molecules, KARs can play the role of co-receptors and thus augment the intensity of the cell response, in particular faced with small quantities of antigens, maintain the cell response over time, and also cooperate in the stimulation of cell proliferation.

The role of KARs, naturally expressed on NK and T lymphocyte sub-populations, is not restricted by their own ligands, namely the class I MHC molecules, but extends to the equilibrium of the immune system generally.

The functioning of naturally expressed KARs therefore influences the proliferation of NK and T cells, the production by these cells of cytokine type substances, the lysis of target cells such as deleterious autologous cells, malignant cells or cells infected by viruses, allogenic cells, but also the tolerance of the immune system faced with certain antigens.

Any non- or dys-functioning of KARs can therefore lead to different diseases or undesired reactions, all associated with the functioning of the immune system, such as immuno-deficiency diseases, auto-immune diseases (*e.g.* multiple sclerosis), tumors, viral, bacterial, parasitic and allergies, graft rejections. For example, it has been shown that if a person only displays less than 10% of lymphocytes expressing KARs, nearly all the lymphocytes of patients suffering from LDGL (lymphoproliferative disease of the granular lymphocytes) express KARs.

A purpose of the present invention is to provide means allowing the diagnosis of an abnormal or undesired functioning of activatory receptors for class I MHC molecules such as KARs and to control their functioning.

Thus a subject of the invention is new polypeptides, called below KARAP (*KAR-Associated Proteins*), which are necessary for transducing a signal originating for a KAR, as well as the antibodies and nucleic acids obtained from said new polypeptides. A subject of the invention is also a process for obtaining said new polypeptides as well as their biological applications.

By "KAR receptor", is meant, in the present invention, human receptors of immunoglobulin type which are non-inhibitory counterparts of KIR receptors, such as KAR p50 (KIRIIDS1 to KIRIIDS5), KIRIIIDS1 receptors, but also non-inhibitory receptors with a similar structure to these KAR receptors, and in particular human receptors of lectin type such as NKG2C, NKG2D (naturally expressed on NK and T cells), murine receptors of immunoglobulin type such as pir A (naturally expressed on myeloid cells, B cells), gp49A (naturally expressed on mastocytes), murine receptors of lectin type such as Ly49D, Ly49H (naturally expressed on NK and T cells).

By KARAP polypeptide is therefore meant any isolated polypeptide (other than a KAR) in the absence of which said KAR receptor is naturally incapable of transducing a dtectable activatory signal. This does not exclude the fact that a determined KARAP polypeptide may not only be associated with a KAR receptor as defined above, but also with other activatory or non-inhibitory monomeric receptors with a structure close to that of KARs as defined above, and in particular with a human activatory receptor of immunoglobulin type of the LIR/MIR/ILT family such as ILT1.

The term "polypeptide" includes, in the present Application, not only said polypeptide, but also the homologues of this polypeptide, as obtained by deletion, insertion, inversion or preservative substitution of amino acids, and the fragments of this polypeptide, as obtained by hydrolysis of said polypeptide using proteases, said homologues or fragments being capable of transducing a signal

originating a KAR. This term "polypeptide" covers, in the present Application, both polypeptides and proteins.

A polypeptide according to the invention is necessary for transducing the signal received by a KAR receptor: therefore it is an isolated polypeptide which allows the restoration of a deficient KAR activation. In order to determine whether a given isolated polypeptide allows the restoration of a deficient KAR activation, a person skilled in the art can proceed by showing that a KAR receptor exists which, if it is expressed by an appropriate cell in the absence of this polypeptide, does not succeed in transducing a detectable activatory signal, or does not succeed in transducing an activatory signal which is satisfactory for the envisaged application. A version of this determination is presented in Example 3 below by comparison between the activation capacity (release of serotonin) of an RBL-2H3 cell expressing the KAR p50.2 receptor only, and that of an RBL-2H3 cell which at one and the same time expresses the KAR p50.2 receptor and its KARAP polypeptide. Examples of appropriate cells are presented in Figure 5 below.

By "restored deficient KAR activation KAR ", is meant that the transduction at the cell, of a significant activatory signal, by said KAR is possible, or, if appropriate, satisfactory. In particular, this can be tested using cellular stimulation by antibodies.

In order to determine at the level of a cell whether a signal originating from a KAR is or is not transduced, and to determine whether such a signal is stimulated or inhibited, numerous means are at the disposal of a person skilled in the art. Exemples of such means include the stimulation of said KAR by a ligand and measurement of the cytokines secreted (*cf.* for example, Cambiaggi *et al.* 1996, Blood 87:2369), of cell proliferation (*cf.* for example Mandelboim *et al.* 1996, Science 274:2097), of cytotoxicity (*cf.* for example the redirected cytotoxicity test described below), of mobilization of intracytoplasmic calcium (*cf.* for example Blery *et al.*, 1997, J. Biol. Chem. 272, 8989-8996), and/or of the induction of phosphorylation (*cf.* for example Vivier *et al.* 1991, J. Immunol. 146:206).

A polypeptide according to the invention is in addition characterized in that it is capable of associating with a KAR, and of not associating with the inhibitory counterpart of this KAR.

Methods allowing determination of whether a polypeptide is capable of associating with a KAR, and of not associating with the inhibitory counterpart of this KAR (i.e. of not associating with a corresponding KIR receptor), are well known to a person skilled in the art. An example of such a method comprises in particular:

- expression of this polypeptide at a KAR^+ KIR^- cell on the one hand, and at a KAR^- KIR^+ cell,

- immunoprecipitation of one or more polypeptide fraction(s) from the lysate of these cells with at least anti-KAR and/or anti-KIR antibodies,

- observation of the presence of said polypeptide in the fraction(s) originating from the KAR^+ KIR^- cell, and the absence of this same polypeptide from the fraction or fractions originating from the KAR^- KIR^+ cell. Examples of anti-KIR and/or anti-KAR antibodies include anti-CD158, anti-p70/NKB1, anti-p140 antibodies and more particularly the EB6, GL183 or PAX250 monoclonal antibodies. A method allowing the expression of such a polypeptide by a cell is indicated in Example 3 below.

A KARAP polypeptide according to the invention can moreover be characterized in that it is obtained:

i. by immunoprecipitation of one or more polypeptide fractions of lysates of cells expressing KAR receptors capable of transducing an activatory signal, with the help of one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

ii. it being possible for each polypeptide fraction optionally to be exhausted beforehand by removal of the fractions immunoprecipitated with the help of anti-CD3ζ and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with the help of one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1, anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

iii. by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight, and recovery of the polypeptides corresponding to a molecular weight of about 12 ± 2 kDa, or

by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight having subjected said polypeptide fraction(s) to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of about 12, 14 and/or 16 ± 2 kDa. The kinase test can be carried out as described below in the examples (cf. material and methods of Example 1 below).

Said cells expressing KAR receptors capable of transducing an activatory signal can be in particular NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes. Means for determining whether a KAR is capable or not of transducing a signal to the cell have been indicated above.

A KARAP polypeptide according to the present invention is, in addition, characterized in that its amino acid sequence:

- has at least one phosphorylatable tyrosine amino acid,

- has a molecular weight comprised between about 10 ± 2 and 16 ± 2 kDa (in particular, real molecular weight of 10 ± 2 kDa, apparent molecular weight on polyacrylamide gel under denaturing conditions of 12 ± 2 to 16 ± 2kDa according to the degree of phosphorylation).

In addition it is characterized in that its amino acid sequence comprises at least one ITAM YxxL/Ix_{6-8}YxxL/I unit in the intracytoplasmic region.

According to one aspect of the invention, the amino acid sequence of a KARAP polypeptide comprises an extracytoplasmic region, a transmembrane region, and/or a entracytoplasmic region. In a characteristic manner, this intracytoplasmic region is in the majority relative to the other regions of the sequence of this polypeptide. Means for identifying the extracytoplasmic, transmembrane, intracytoplasmic regions are known to a person skilled in the art (for example, hydropathicity algorithms, formation of inverse vesicles).

According to another aspect of the invention, the amino acid sequence of a KARAP polypeptide contains at least one extracytoplasmic cysteine amino acid.

According to yet another aspect of the invention, the amino acid sequence of a KARAP polypeptide contains at least one transmembrane charged amino acid (R, K, D, E).

The polypeptides according to the invention can be phosphorylated at the level of at least one tyrosine residue, or be non phosphorylated.

In one embodiment of the invention, said polypeptides are presented in the form of dimers linked by a disulphide bridge; they associate in a selective and non covalent manner with KARs which function, either as autonomous receptors for class I MHC molecules, or as co-receptors of TCR or of an RFc such as CD16.

According to an advantageous aspect of the invention, a KARAP polypeptide is capable of binding to a molecule having an SH2 domain such as ZAP-70, $p72^{syk}$, $p56^{lck}$, $p59^{fyn}$, $p60^{lyn}$, Grb-2, pp36-38 (lat), PLC-α1, p85 (PI-3 kinase), Shc, or to a molecule having a PTB domain (*PhosphoTyrosine Binding*) such as Shc. Such a binding can be observed by incubation of polypeptides according to the invention with molecules having an SH2 or PTB domain and measurement of the plasmon resonance (Olcese *et al.* 1996, The Journal of Immunology 156:4531-4534).

A particular KARAP polypeptide according to the invention has an amino acid sequence essentially constituted by SEQ ID n°2. The present invention also relates to polypeptides the sequence of which is essentially constituted by the extracytoplasmic part of SEQ ID n°2, namely SEQ ID n°3, or by the transmembrane part of SEQ ID n°2, namely SEQ ID n°4, or the intracytoplasmic part of SEQ ID n°2, namely SEQ ID n°5. Other particular KARAP polypeptides

according to the invention have an amino acid sequence essentially constituted by SEQ ID n°11, n°12, n°13, n°14, n°15, n°17 (consensus sequence of the KARAP protein of mouse C57Bl/6), or n°28 (protein sequence of the KARAP of mouse 129 obtained from the genomic sequence).

Such polypeptides can also be obtained, after sequencing, by chemical synthesis or using recombinant DNA techniques.

Said KARAP polypeptides are necessary for transducing signals originating from activatory receptors, the KARs, which have neither intracytoplasmic ITIM nor ITAM but which have a transmembrane amino acid residue.

According to an advantageous provision, the polypeptides according to the invention are modified by glycosylation, phosphorylation, sulphonation, biotinylation, acylation, esterification, or by addition, substitution or suppression of entities having a molecular shape similar to that of phosphate groups, such as phosphonate, by the addition of tracer reagents such as luciferase, GFP (*Green Fluorescence Protein*) or analogues thereof, by the addition of purification targets such as an affinity ligand, or by the addition of entities modifying its solubility. Modifications of particular benefit include those which modify said polypeptide so as to block or inhibit its capacity to transduce the signal received (negative transdominant strategy). A polypeptide according to the invention, in a form modified in this way, is used in particular in any composition or method intended to modulate in a negative manner (inhibit) a given immune response, in particular an undesired or abnormal immune response (for example, autoimmune diseases, allergies, graft rejection). Thus appropriate modifications include those which render the phosphorylation on tyrosine of said polypeptide non hydrolysable under biological conditions (for example, by the addition of phosphonate groups). They also include those which render non functional an amino acid residue which is critical to the functioning of a polypeptide according to the invention: for example, by substitution or mutation of a tyrosine residue (Y), in particular a tyrosine residue contained in an ITAM unit, into a phenylalanine residue (F), which hinders the binding of said polypeptide thus modified to a protein having an SH2 or PTB domain.

According to another advantageous provision, the polypeptides of the invention, their fragments, homologues, or modified forms are capable of crossing a cell membrane, i.e. double lipidic layer.

The present invention also relates to antibodies, in particular monoclonal antibodies, and the fragments of such antibodies, in particular the Fc, Fv, Fab, $F(ab)'_2$, CDR fragments, as obtained by immunogenesis from a KARAP polypeptide according to the invention, or as obtained from a fragment, homologue or modified form of such a polypeptide.

In particular a subject of the invention is fragments of such antibodies, in particular an Fc, Fv, Fab, F(ab)'$_2$, CDR fragment, as obtained by immunogenesis from a polypeptide the sequence of which is essentially constituted by the extracytoplasmic, intracytoplasmic, or transmembrane part of such a KARAP polypeptide according to the invention. In particular it relates to antibodies capable of recognising, according to an antigen-antibody type reaction, SEQ ID n°2, SEQ ID n°3, SEQ ID n°4, SEQ ID n°5, SEQ ID n°11, SEQ ID n°12, SEQ ID n°13, SEQ ID n°14, SEQ ID n°15, SEQ ID n°17 and/or SEQ ID n°28, as well as their fragments.

Such antibodies are obtained by the immunization of animals, such as rabbits and mice, against polypeptides, fragments, homologues or forms modified according to the invention as essentially obtained by elution of electrophoretic bands, by chemical synthesis or by a soluble fusion protein technique (GST), said polypeptides, fragments, homologues or modified forms being optionally coupled to immunogens such as ovalbumin.

Monoclonal antibodies are then produced by hybridomal fusion of lienal immune cells, screening and purification of the culture supernatants (Köhler and Milstein, 1975, Nature 256, 495-497; Antibodies, a laboratory manual, 1988, Harlow and David Lane, Ed. Cold Spring Harbor laboratory).

From these antibodies, diantibodies can be generated according to standard procedures. Said fragments can, if necessary, be inserted in or grafted to humanizing structures.

The present invention also relates to the nucleic acids containing a sequence corresponding to the open reading frame, according to the universal genetic code, and taking into account the degeneration of said code, the amino acid sequence of a polypeptide, fragment, or homologue according to the invention, as well as the variants which have a homology greater than or equal to 60% with such nucleic acids, and which are capable of coding for a molecule transducing an activatory signal originating from a KAR as defined above. In particular it relates to any nucleic acid the DNA sequence of which is essentially constituted by SEQ ID n°1 (cDNA of the mature KARAP protein of sequence SEQ ID n°2), n°6, n°7, n°8, n°9, n°10, n°16 (consensus cDNA sequence of KARAP of mouse C57Bl/6), n°27 (cDNA sequence of KARAP of mouse 129 obtained from the genomic sequence), n°18 (genomic sequence of KARAP of mouse 129), or n°31 (cDNA sequence of human KARAP), or by any part corresponding to the extra-, intra-cytoplasmic and/or transmembrane regions of these sequences, or by any part corresponding to an exon or an intron of these sequences.

The present invention also relates to a process for obtaining a polypeptide according to the invention comprising the following stages:

i. immunoprecipitation of one or more polypeptide fraction(s) of lysates of cells expressing functional KAR receptors (for example NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes) using one or more anti-KIR and/or anti-KAR antibodies, such as an anti-CD158, anti-p70/NKB1, anti-p140 antibody, and more particularly the monoclonal EB6, GL183 or PAX250 antibodies,

ii. it being possible for each polypeptide fraction optionally to be exhausted beforehand by removal of the fractions immunoprecipitated with the help of anti-CD3 and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with the help of one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1, anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

iii. separation of the polypeptides from said polypeptide fraction(s) according to their molecular weight and recovery of the polypeptides corresponding to a molecular weight of approximately 12 ± 2 kDa, or

separation of the polypeptides of said polypeptide fraction(s) according to their molecular weight having subjected said polypeptide fraction(s) to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of approximately 12, 14 and/or 16 ± 2 kDa.

A subject of the present Application is also a method for obtaining the sequence of particular KARAP polypeptides according to the invention. This method, a version of which is described in Example 2 below (bio-informatics strategies), includes in particular the screening of those of the polypeptide sequences which correspond to the following criteria:

- the sequence has at least one phosphorylable tyrosine amino acid,
- the sequence has a molecular weight between approximately 5 and 25 kDa,
- the sequence comprises an extracytoplasmic region, a transmembrane region, and an intracytoplasmic region,
- the sequence has at least one cysteine amino acid in its extracytoplasmic region,
- the sequence includes at least one charged amino acid (R, K, D, E) in its transmembrane region, and
- the sequence includes at least one ITAM YxxL/Ix_{6-8}YxxL/I unit in its intracytoplasmic region,
- the polypeptide corresponding to the selected sequence must be capable of associating with a KAR, and not associating with the corresponding inhibitory counterpart receptor (KIR), as defined above.

A subject of the present Application is also a method for determining or checking whether a candidate polypeptide corresponds to a KARAP polypeptide according to the invention. An embodiment of such a method is given in Example 2 below. Such a method consists of producing an antibody against a characteristic part of this candidate polypeptide (for example an intracytoplasmic region comprising at least one ITAM unit or an extracytoplasmic region), and to check that a KAR receptor exists which, when it is expressed functionally on a cell, is combined with a recognized element, according to a reaction of antigen-antibody type, by said antibody.

This method, according to the invention, of identifying KARAP polypeptides thus consists in particular of:

- producing a mono- or polyclonal antibody directed against this candidate polypeptide, and in particular against an extracytoplasmic region of this candidate polypeptide and/or a region which comprises at least one ITAM unit (for example, in the case of the mouse KARAP protein SEQ ID no. 2 identified above, an antibody directed against a region of the extracytoplasmic part (SEQ ID no. 3) or of the intracytoplasmic part (SEQ ID no. 5) of SEQ ID no. 2),
- brining this antibody into contact with a lysate of cells, possessing, in a functional form, the activatory or non-inhibitory receptor for which the candidate polypeptide is supposed to continue the KARAP, under mild conditions allowing binding reactions of antigen-antibody type,
- identifying the candidate polypeptide as being a KARAP polypeptide according to the invention when, in any reaction products formed, there are a product having an apparent molecular weight close to that of said activatory or non-inhibitory receptor (approximately 50 kDa for the KAR p50) and a product having an apparent molecular weight close to that of the candidate polypeptide (in particular between approximately 10 and 16 kDa).

This identification method according to the invention can in particular be carried out:

- by bringing said antibody into contact as described above,
- precipitating any reaction products formed under mild detergent conditions maintaining the molecular complexes (for example 1% digitonin, see Example 1 above),
- measuring the molecular weight of the precipitated products, for example by electrophoretic migration in the presence of the markers of molecular weight on a polyacrylamide gel under denaturing conditions, and
- identifying the candidate polypeptide as being a KARAP polypeptide according to the invention as described above.

The present invention also relates to a pharmaceutical composition comprising, in conjunction with a pharmaceutically acceptable vehicle, an effective quantity of at least one polypeptide, KARAP, fragment, homologue or form modified according to the invention, at least one antibody or fragment of antibody according to the invention, or at least one nucleic acid or nucleic acid variant according to the invention.

The pharmaceutical composition according to the invention can be formulated in solid or liquid form or in the form of a suspension, for oral, parenteral, topical, intravaginal, intrarectal administration or for oral and/or nasal inhalation.

Said pharmaceutical composition according to the invention is intended to modulate the activity of a KAR. In order to stimulate the activity of a KAR, said pharmaceutical composition will comprise agents facilitating the transduction of the signal originating from said KAR, such as, for example, polypeptides, fragments, homologues, or nucleic acids, variants according to the invention capable of crossing a double lipidic layer. In order to inhibit the activity of a KAR, said pharmaceutical composition will comprise agents blocking the transduction of the signals originating from said KAR such as, for example, fragments of antibodies according to the invention capable of crossing a double lipidic layer in order to block the cellular KARAPs, or modified polypeptides, according to the invention, phosphorylated or not, for example by phosphorylation not hydrolysable under biological conditions, in order to block proteins with an SH2 (ZAP-70, $p72^{syk}$) or PTB domain or any molecule which adapts or carries out the activation of said KAR. Such modifications include in particular the addition of phosphonate groups, and/or the mutation of at least one Tyrosine residue (Y) into a phenylalanine residue (F).

The present Application therefore relates to a composition for the prevention, the reduction, and/or the treatment of an abnormal or undesired functions of a cell involved in an immune reaction. Such a composition advantageously includes polypeptides, or, if appropriate, modified polypeptides according to the invention.

In order to determine at the level of a cell whether a signal originating from a KAR is or is not transduced, and to determine whether such a signal is stimulated or inhibited, numerous means are at the disposal of a person skilled in the art. Examples of such means have been indicated above.

The use of said polypeptides, antibodies and nucleic acids as diagnostic agents, also falls within the scope of the present invention (diagnostic methods, and diagnostic kits permitting their implementation).

The present invention also relates to a method of *in vitro* diagnosis of abnormal or undesired functioning of a cell, comprising the following stages:

- bringing at least one cell, or cell extract, into contact with an antibody according to the invention, or a fragment of such antibody, or with a nucleic acid according to the invention or a variant of such nucleic acid, and
- revealing any reaction product formed.

The stage of bringing into contact is carried out under conditions in particular of duration, temperature, buffer, where appropriate gel crosslinking, allowing the establishment of a reaction of antigen-antibody type for example by ELISA (*Enzyme Linked Immunoabsorbent Assay*), or where appropriate, of a reaction of nucleic acids hybridization and PCR type (polymerase chain reaction).

For the revelation of any reaction product formed, tracers can be used such as fluorescent, enzymatic, radioactive or luminescent tracers.

Said *in vitro* diagnostic method according to the invention allows the diagnosis of abnormal or undesired cellular functioning which can manifest themselves as an immunoproliferative disease, an immunodeficiency disease such as an HIV disease, a cancer such as lymphoproliferative disease of the granular lymphocytes, an auto-immune disease such as rheumatoid arthritis, an infectious disease such as malaria, an allergic response, a transplant rejection.

The present invention also relates to a method for identifying molecules which adapt or carry out the activation of a KAR, and to a method for identifying molecules capable of modulating a cell activity resulting from the activation of a KAR.

Said method for identifying molecules which adapt or carry out the activation of a KAR according to the invention comprises the following stages:

i. bringing the candidate molecules into contact with the polypeptides according to the invention (or with fragments or homologues of such polypeptides), and

ii. selecting those candidate molecules for which a binding with said polypeptides (or with said fragments of polypeptides) is observed.

The candidate molecules likely to be molecules which adapt or carry out the activation of a KAR can be for example chosen from the molecules with an SH2 or PTB domain. These can be in soluble recombinant form.

The stage of bringing into contact can be, for example, carried out by coupling the candidate molecules, obtained in soluble recombinant form, likely to be molecules which adapt or carry out the activation of a KAR, to balls allowing the measurement of radioactivity such as balls of scintillating liquid, and by passing polypeptides according to the invention (or fragments or homologues of such polypeptides) in tritiated form over said balls. Those candidate molecules for

which a binding to said polypeptides, fragments, or homologues is observed by measuring the radioactivity (cpm) are then selected.

The stage of bringing into contact can also be carried out by immobilization of polypeptides according to the invention (or fragments or homologues of such polypeptides) on microsupports allowing the measurement of the plasmon resonance such as BIAcore microsupports (Pharmacia) (*cf.* for example Olcese *et al.*, 1996, The Journal of Immunology 156:4531-4534; Vely *et al.*, Immunology Letters 1996, vol. 54, p145-150), or by immobilization of phosphorylated and biotinylated polypeptides according to the invention on streptavidine balls (Vély *et al.* Eur. J. Immunol. 1997, 27: 1994-2000; Le Dréan *et al.* Eur. J. Immunol. 1998, 28: 264-276), and by passing, over said microsupports, candidate molecules likely to be molecules which adapt or carry out the activation of a KAR. Those candidate molecules for which a binding to said polypeptides, fragments, or homologues is observed by measuring the plasmon resonance (Resonance Unit) are then selected.

This method of identifying the molecules which adapt or carry out the activation of a KAR, whatever its implementation method, can also be used as a reference for the implementation of the method for identifying molecules capable of modulating a cell activity resulting from the activation of a KAR according to the invention.

This method of identifying molecules capable of modulating a cell activity resulting from the activation of a KAR, according to the invention, comprises the following stages:

i. bringing the candidate molecules into contact with molecules which adapt or carry out the activation of a KAR as obtained by the method according to the invention described above and with polypeptides according to the invention (or with fragments or homologues of such polypeptides), and

ii. selection of those candidate molecules which have an effect on the binding between said polypeptides (or said fragments or homologues of polypeptides) and said molecules which adapt or carry out the activation, as observed in the absence of said candidate molecules.

The candidate molecules likely to modulate a cell activity resulting from a KAR can be chosen from banks of natural or synthetic compounds, in particular from chemical or combinatory banks. Said candidate molecules can be of protein nature (for example, derivatives or fragments of anti-idiotype antibodies such as the antibodies according to the invention, derivatives or fragments of catalytic antibodies), of carbonated, lipidic or nucleic nature.

The bringing-into-contact stage of the method for identifying molecules capable of modulating a cell activity resulting from the activation of a KAR,

according to the invention, can be, for example, carried out by incubation of said candidate molecules with polypeptides according to the invention (or with fragments or homologues of such polypeptides) and with molecules which adapt or carry out the activation of a KAR, as obtained by the method according to the invention, under conditions allowing measurement of the degree of binding between said polypeptides and said molecules which adapt or carry out the activation of a KAR, for example, based on a chemical property of said molecules which adapt or carry out the activation in a non-bound state, such as an enzymatic property, phosphorylation or self-phosphorylation property.

The bringing-into-contact stage of the method for identifying molecules capable of modulating a cell activity resulting from the activation of a KAR, according to the invention, can also be carried out by implementing techniques of the scintillating liquid balls type and tritiated polypeptides or polypeptides of microsupport type and measurement of the plasmon resonance, as described above, by measuring the radioactivity or, respectively, the plasmon resonance, resulting from the binding between said polypeptides and said molecules which adapt or carry out the activation, in the absence and in the presence of candidate molecules. Those candidate molecules which either increase or decrease in a statistically significant manner the control degree of binding measured between said polypeptides and said molecules which adapt or carry out the activation in the absence of said candidate molecules are then selected.

The molecules capable of modulating the activation of a KAR, as identified by the method according to the invention, can be modified chemically in order to render them non-hydrolysable under biological conditions, and/or so that they can cross a double lipidic cell layer.

The molecules capable of modulating the activation of a KAR, according to the invention, advantageously act by modifying the interaction between said KARAPs and their cellular effectors or adaptors.

Said molecules capable of modulating a cell activity resulting from the activation of a KAR, according to the present invention, can then be applied to a cell cultivated *in vitro*, such as a lymphocyte cell, of which the KAR activity has been stimulated, for example, by bringing it into contact with a ligand. This application is achieved by penetration inside said cell, for example, by electroporation or by chemical modification allowing a double lipidic layer to be crossed.

The present invention is illustrated by the following examples which should be in no event be considered as limitative.

Reference is made to the 23 following figures:

- Figure 1 shows

in A, an analysis by flow cytometer (FACScan, registered trade mark of Becton-Dickinson) in indirect immunofluorescence of cells cultivated on IL-2 (interleukin 2) and from patients suffering from LDGL (lymphoproliferative disease of the granular lymphocytes) designated R.P., D.F. and MAL., and

in B, the results of a re-directed cytotoxicity test with different monoclonal antibodies, carried out on NK cells cultivated on IL-2 from different donors;

- Figure 2 shows:

in A, an SDS-PAGE analysis (resolution of proteins by electrophoresis on gel and sodium dodecyl sulphate) carried out from NK cells of donor R.P. ($p50.1^+$) radiomarked with ^{125}I and immunoprecipitated using the monoclonal anti-CD158 EB6 antibody, before and after exhaustion of FcεRIγ and of CD3ζ using anti-CD3ζ / anti-FcεRIγ antibodies,

in B, an SDS-PAGE analysis with an anti-CD3ζ antibodies probe of full lysates of D.F. cells or of immunoprecipitates of such lysates;

- Figure 3 shows:

in A, SDS-PAGE analysis of the phosphorylated proteins originating from *in vitro* kinase tests to which immunoprecipitates of lysates of NK MAL. cells have been subjected,

in B, the same type of SDS-PAGE analysis as in Figure 3A but carried out from RBL-2H3 $p50.2^+$ cells,

in C, an analysis by thin-layer electrophoresis (TLE) of the phosphorylated amino acids of the KARAPs and CD3ζ bands excised after *in vitro* kinase tests carried out on anti-CD158 and anti-CD16 immunoprecipitates, respectively, of NK R.P. cells,

- Figure 4 shows a 2-dimensional SDS-PAGE analysis under non-denaturing/denaturing conditions of anti-CD158 immunoprecipitates of lysates of cells NK R.P. having undergone a kinase test, and

- Figure 5 shows the activatory or non-inhibitory receptors of the immunoglobulins superfamily (IgSF) or of lectin type, and their inhibitory counterparts,

- Figure 6 shows the schematic structure of KIR (p58) and KAR (p50) receptors,

- Figure 7 shows the cDNA sequence of a mouse KARAP polypeptide according to the invention (SEQ ID no. 1),

- Figure 8 shows the nucleotide sequence (comprised between the excluded leader sequence and the stop codon) and the amino acids sequence of a KARAP polypeptide according to the invention (mature protein, SEQ ID no. 2), and

- Figure 9 shows the alignment of the ITAMs and of the ITAM of a KARAP polypeptide according to the invention,

- Figures 10A, 11A, 12A, 13A and 14A respectively illustrate the cDNA sequences of the EST's AA242315, AA734769, W88159, AA098506 and W41142 (SEQ ID no. 6 to SEQ ID no. 10),

- Figures 10B, 11B, 12B, 13B and 14B respectively illustrate the protein sequences of the EST's AA242315, AA734769, W88159, AA098506 and W41142 (SEQ ID no. 11 to SEQ ID no. 15),

- Figure 15 shows the alignment of the cDNA sequences of the EST's AA242315, AA734769, W88159, AA098506 and W41142, and the resulting consensus sequence (SEQ ID no. 16; KARAP consensus cDNA of mouse C57Bl/6),

- Figure 16 represents the alignment of the protein sequences of the EST's AA242315, AA734769, W88159, AA098506 and W41142, and the resulting consensus sequence (SEQ ID no. 17; KARAP consensus protein of mouse C57Bl/6),

- Figure 17 shows the sequence of the KARAP gene of a mouse of line 129 (SEQ ID no. 18; 2838 pb),

- Figure 18 shows the genomic organization of the KARAP of a mouse of line 129,

- Figure 19 shows the cDNA sequence of the KARAP of a mouse of line 129 (SEQ ID no. 27) and the corresponding protein sequence (SEQ ID no. 28),

- Figure 20 represents from top to bottom the genomic organization of the KARAP gene of a mouse of line 129, the corresponding protein sequence, and the nature of the differents regions of this protein,

- Figure 21 shows the cDNA of the human KARAP (SEQ ID no. 31),

- Figure 22 shows the percentage of serotonin salted out in the supernatant by the doubly transfected p50/human KARAP RBL-2H3 cells, and stimulated by the antibody indicated on the abscissa (on the left: no antibodies; in the centre: mouse IgE: mIgE 1/500; on the right: GL183 5µg/ml),

- Figure 23 illustrates the homology between the organization of the human KARAP gene and that of the murine KARAP gene.

EXAMPLE 1:

1. Materials and methods

Monoclonal antibodies (mAbs) and reagents

The following monoclonal antibodies were used:

- anti-CD3, anti-CD16 and anti-CD56 antibodies of isotype IgG1, such as JT3A (Coulter Immunotech reference 0178), KD1 (Coulter Immunotech reference 0813) and TA181.H12 (Coulter Immunotech reference 1844), respectively,

- anti-CD3ζ antibodies such as TIA-2 (Coulter Immunotech 66045P2),

- anti-CD158 antibodies, namely anti-p58.1 antibodies such as EB6 (Coulter Immunotech reference 1847), anti-p58.2 antibodies such as GL183 (Coulter Immunotech reference 1846) and anti-p50.3 antibodies such as PAX250 described in Bottino *et al.* (*Eur. J. Immunol.,* 1996, 26, 1816),

- an anti-FcεRIγ rabbit antiserum such as antiserum 666 described in Jouvin M.H. *et al.*, 1994, J. Biol. Chem., 269, 5918-5925,

- an anti-FcεRIα rabbit antiserum such as antiserum BC4 described in Bociano L.K. *et al.*, 1986, J. Biol. Biochem., 261, 11823-11831,

- an anti-mouse goat antiserum conjugated with horseradish peroxidase (Sigma A-2304) and an anti-rabbit goat antiserum conjugated with horseradish peroxidase (Sigma A-0545),

- an anti-mouse goat immunoglobulin conjugated with fluorescein isothiocyanate (Coulter Immunotech 0819 $F(ab')_2$) and

- GL183-phycoerythrin (GL183-PE) monoclonal antibodies (Coulter Immunotech 2278), EB6-phycoerythrin (EB6-PE) monoclonal antibodies (Coulter Immunotech 2277) and an anti-mouse-phycoerythrin (anti-mouse-PE) goat immunoglobulin (Coulter Immunotech 0855 $F(ab')_2$).

The lysis buffer contained Tris-HCl 25 mM pH 7.5; NaCl 150 mM; digitonin 1%; sodium orthovanadate 100 µM; NaF 10 mM; aprotinin 2 µg/ml; leupeptin 2 µg/ml; all these products were purchased from Sigma (St Louis, MO, USA).

The kinase buffer contained Hepes 20 mM pH 7.2; NaCl 100 mM; $MnCl_2$ 5 mM; $MgCl_2$ 5 mM; $^{32}\gamma$ ATP 10 µCi = 370 kBq (Amersham, Buckinghamshire, UK).

The thin layer electrophoresis (TLE) buffer contained 10% glacial acetic acid and 1% pyridine in water; pH 3.5.

Cells

Human NK cells from LDGL patients, or LDGL cells

The human NK cells were obtained from patients suffering from lymphoproliferative disease of granular lymphocytes (LDGL) of the $CD56^+$, $CD16^+$, $CD3^+$ NK cell line. Peripheral blood lymphocytes (PBL) were isolated from blood samples of patients suffering from LDGL by Ficoll/Hypaque gradient

centrifugation. These LDGL cells were then cultivated at 37°C at a concentration of 10^6 cells per ml on RPMI-1640 medium containing 10 µg/ml of penicillin-streptomycin and 10% of foetal calf serum, in the presence of allogenic irradiated nurse cells and 100 U/ml of rIL-2.

Preparation of RTIIB.p50.2$^+$ transfected cells

Transfectants of RBL-2H3 cells (American Type Culture Collection) expressing p50.2 KARs (RTIIB.p50.2$^+$ cells) were prepared as described in Bléry *et al.*, 1997, J. Biol. Chem., 272, 8989-8996. Figure 6 schematically shows the structure of p58 KIRs (immunoglobulin-type inhibitory human receptors) and p50 KARs (non-inhibitory counterpart of p58 KIRs).

In brief, the RTIIB cells used are the cells conventionally described as being RBL-2H3 cells transfected so as to express the murine FcγRIIb2 receptor and the CD25/CD3 chimeric molecule comprising the complete ectomembrane and transmembrane domains of human CD25 bound to the complete intracytoplasmic domain of murine CD3.

These RTIIB cells were also transfected, by electroporation, with 183.Act2 cDNA (coding for p50.283) carried on expression vector RSV-5gpt.

Stable RTIIB.p50.2$^+$ transfected cells were established by culture in the presence of xanthine (250 µg/l), hypoxanthine (13.6 µg/l) and mycophenolic acid (2 µg/l).

Cytolytic test

The cytolytic activity of LDGL cells cultivated on IL-2 was measured relative to the P815 murine cell line (American Type Culture Collection) in the absence or presence of anti-CD16, anti-CD158 and anti-CD56 mAbs.

In brief, 5 x 10^3 target cells labelled with ^{51}Cr were added to serial dilutions of effector cells in the presence of 50 µl of hybridoma supernatant monoclonal antibody at the start of the standard ^{51}Cr release test lasting 4 hours (Vivier E. *et al.*, 1991, *J. Immunol., 146,* 206).

Radioiodination

The cells (10 - 50 x 10^6) were fixed with 0.5% formaldehyde in PBS (sodium phosphate buffer) and then permeabilized for 5 minutes with digitonin at a concentration of 30 µg/ml in PBS, prior to iodination catalyzed by

lactoperoxidase (^{125}I, NEN-Dupont, Wilmington, DE, USA), as described by Anderson P. *et al.*, 1989, *J. Immunol., 143,* 1899.

The cells were lyzed for 30 minutes at 4°C in a digitonin lysis buffer. The prepurified postnuclear supernatants were then immunoprecipitated with specific antibodies covering S4B-Sepharose beads (Pharmacia, Piscataway, NJ, USA) (Vivier E. *et al.*, 1991, *J. Immunol., 146*, 206). The immunoprecipitates were analyzed by SDS-PAGE (protein resolution by electrophoresis on gel and sodium dodecylsulfate) and autoradiography.

***In vitro* kinase test**

The cells (10 x 10^6 per sample) were lyzed in 1 ml of lysis buffer (cf. reagents). The prepurified postnuclear supernatants were immunoprecipitated for 2 to 3 hours using monoclonal antibodies covalently bonded to a Sepharose 4B activated by CnBr (Pharmacia). The immune complexes were washed three times in lysis buffer; 40 µl of kinase buffer (cf. reagents) were then added to the immunoprecipitates over 10 minutes at 37°C. The kinase reaction was stopped by the addition of SDS-sample reducing buffer. The samples were brought to the boil prior to analysis by SDS-PAGE and autoradiography. In some experiments, the samples were analyzed by two-dimensional non-denaturing/denaturing diagonal SDS-PAGE.

Analysis of the phosphorylation of the KARAPs

After the *in vitro* kinase test and the separation by SDS-PAGE, the phosphorylated proteins were cut out of the dried gels and eluted using a Centrilutor (Amicon) or a 0.1% solution of SDS (sodium dodecylsulfate) in PBS (sodium phosphate buffer). The eluted proteins were precipitated in 20% trichloroacetic acid at 4°C for 2 hours, prior to incubation in 200 µl of 5.7 M HCl at 110°C for 90 minutes. The individual amino acids were then dried and resuspended in 5 µl of TLE buffer (cf. reagents) containing 5 µg each of unlabelled phosphotyrosine, phosphothreonine and phosphoserine (Sigma) as standard references. The samples were deposited on plates of cellulose (100 µm DC cellulose) and caused to migrate at 1500 V for 45 minutes at 4°C on a Multiphor II (Pharmacia). Standard references were developed with 1% ninhydrin in acetone and the ^{32}P-labelled amino acids were identified by autoradiography.

Analysis by immunotransfer

The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose filters and compared with anti-CD3ζ or anti-FcεRIγ antibody probes diluted in PBS solution containing 5% of skimmed dried milk. The immunotransfers were revealed using an anti-mouse or anti-rabbit goat antiserum conjugated with horseradish peroxidase (Sigma references A-2304 and A-0545 respectively) and the ECL detection system marketed by Amersham (RPN 2209).

2. Results

Surface phenotype

The surface phenotype of the NK cells taken from LDGL patients and cultivated on IL-2 (interleukin-2) was analyzed by FACScan (screening of fluorescence-activated cells) using the indirect immunofluorescence method.

The results of the study relating to three of these patients, hereafter called R.P., D.F. and MAL., are reported below.

Said results are illustrated in Figure 1A, which shows an indirect immunofluorescence FACScan analysis of R.P., D.F. or MAL. LDGL (lymphoproliferative disease of granular lymphocytes) cells cultivated on IL-2. An anti-mouse goat immunoglobulin conjugated with fluorescein isothiocyanate was used as the reagent in the second step. For each type of LDGL cells (R.P. LDGL cells for the analyses shown in the top horizontal band, D.F. LDGL cells for those of the middle horizontal band, MAL. LDGL cells for those of the bottom horizontal band) and for each treatment undergone (control treatment C for the graphs shown on the left or treatment with the indicated monoclonal antibody, i.e., from left to right, anti-CD3, anti-CD16, anti-CD158 EB6, anti-CD158 GL183, anti-CD158 PAX250), the fluorescence intensities are plotted on the abscissa and the relative number of cells is plotted on the ordinate.

It can be observed that:

- the R.P., D.F. and MAL. NK cells are all $CD3^+$ and $CD16^+$;
- the R.P. NK cells are $p50.1^+$, $p50.2^+$, $p50.3^+$: they are recognized by the anti-CD158 monoclonal antibody EB6 and are not recognized by the anti-CD158 monoclonal antibodies GL183 and PAX250;
- the D.F. NK cells are $p50.1^+$, $p50.2^+$, $p50.3^+$: they are recognized by the anti-CD158 monoclonal antibody GL183 and are not recognized by the anti-CD158 monoclonal antibodies EB6 and PAX250; and

- the MAL. NK cells are p50.1$^+$, p50.2$^+$, p50.3$^+$: they are recognized by the anti-CD158 monoclonal antibody PAX250 and are not recognized by the anti-CD158 monoclonal antibodies EB6 and GL183.

The three patients suffering from LDGL therefore showed a lymphoproliferation of NK cells which was recognized by anti-CD158 antibodies: anti-p58.1 KIR (EB6), anti-p58.2 KIR (GL183) and anti-p50.3 KAR (PAX250) respectively. Three groups of NK cells could thus be defined: R.P. LDGL cells, D.F. LDGL cells and MAL. LDGL cells.

Cytolytic test

Redirected cytotoxicity tests using P815 as FcγR$^+$ target cells were carried out on the R.P. p50.1$^+$, D.F. p50.2$^+$ and MAL. p50.3$^+$ NK cells.

The results are illustrated in Figure 1B, which shows a redirected cytotoxicity test with different monoclonal antibodies: NK cells taken from the indicated donors (R.P. p50.1$^+$ on the left, D.F. p50.2$^+$ in the centre or MAL. p50.3$^+$ on the right) and cultivated on IL-2 were used as effector cells. The test was carried out in the presence of: no antibody (white circles), anti-CD16 monoclonal antibody (black triangles), anti-CD56 monoclonal antibody (white triangles), anti-CD158 monoclonal antibody (EB6 for R.P., GL183 for D.F. and PAX250 for MAL.) (black circles). The ratios of effector cells to target cells (E:T ratio: 8:1; 4:1; 2:1; 1:1; 0.5:1; 0.25:1) are plotted on the abscissa and the percentage of specific lysis (scale from 0 to 120%) is plotted on the ordinate.

The redirected cytotoxicity tests indicate that, by contrast with what is observed when stimulating KIRs, the addition of anti-CD158 antibodies to the NK cells considerably increases the cytolysis of the P815 cells (Figure 1B).

As controls, the anti-CD16 monoclonal antibodies increase the spontaneous cytolysis of the P815 cells in a manner similar to the anti-CD158 monoclonal antibodies, whereas an anti-CD56 monoclonal antibody matched to the isotype has no effect (Figure 1B).

These NK cells therefore express KARs, the activatory isoform of KIRs, on their surface. These results were further confirmed by PCR (polymerase chain reaction) analyses with KIR/KAR cDNA reverse transcriptase.

Analysis of the expressed KARs by radioiodination and immunotransfers: identification of the KARAPs

The KARs expressed on the NK cells taken from LDGL patients were analyzed by internal radioiodination followed by immunoprecipitation.

The results are illustrated in Figure 2A, which shows an SDS-PAGE analysis on a 13% gel under denaturing conditions, carried out on NK cells (10 x 10^6 cells/lane) from the donor R.P. ($p50.1^+$) which have been radiolabelled with ^{125}I, immunoprecipitated with the anti-CD158 monoclonal antibody EB6 (lane 1), then purified with anti-CD3ζ/anti-FcεRIγ monoclonal antibodies (lanes 2 to 7) and finally re-immunoprecipitated with the anti-CD158 monoclonal antibody EB6 (lane 8).

The same profiles were obtained with the donors D.F. ($p50.2^+$) and MAL. ($p50.3^+$) (data not shown).

It can be seen that the immunoprecipitates of anti-CD158 antibodies prepared from lyzates of NK cells contain, in addition to the KARs observed at ≈50 kDa, a band of lower molecular weight migrating to about 12 ± 1 kDa.

It was shown that KIRs associate with the polypeptides CD3ζ and FcεRIγ in human NK cells. Pre-exhaustion experiments using anti-CD3ζ and anti-FcεRIγ antibodies eliminated the possibility that the band at about 12 kDa associated with the KARs might be CD3ζ or FcεRIγ (Figure 2A).

The group of proteins corresponding to this band at about 12 ± 1 kDa was given the name KARAPs (*KAR-associated proteins*).

These results were confirmed by immunotransfer experiments, which revealed the absence of any reactive band in the presence of anti-CD3ζ antibodies in the immunoprecipitates of anti-CD158 mAbs prepared from NK lyzates.

These results, obtained in the presence of anti-CD3ζ antibodies, are illustrated in Figure 2B, which shows an analysis of complete lyzates of D.F. cells, or immunoprecipitates of such lyzates, by SDS-PAGE resolution on a 15% gel under denaturing conditions and incubation of the nitrocellulose filters with an anti-CD3ζ monoclonal antibody probe (marker arrow on the right). The complete lyzates of D.F. cells (CCL) were deposited at the rate of 5 x 10^6 cells/lane in lane 1 and the immunoprecipitates of such lyzates were deposited at a rate of 15 x 10^6 cells/lane in lanes 2 to 4. The immunoprecipitations were carried out on lyzates of D.F. cells using the anti-FcεRIα monoclonal antibody BC4 in control lane 2, the anti-CD16 monoclonal antibody in lane 3 and the anti-CD158 monoclonal antibody GL183 in lane 4.

The same results were obtained for the R.P. and MAL. cells with anti-CD3ζ mAb.

The results obtained with anti-FcεRIγ mAb (data not shown) provided the same confirmation.

Analysis of the KARAPs by an *in vitro* kinase test and thin layer electrophoresis (TLE)

In vitro kinase tests carried out on the immunoprecipitates of anti-CD158 monoclonal antibodies revealed that the KARs associate with a predominant phosphoprotein of low molecular weight migrating to about 14 ± 1 kDa in the NK cells.

The results are illustrated in Figure 3A: lyzates prepared from MAL. NK cells were immunoprecipitated with the indicated antibody (anti-FcεRIα in lane 1, anti-CD16 in lane 2, anti-CD158 in lane 3) prior to *in vitro* kinase tests. The phosphorylated proteins were separated by SDS-PAGE on a 15% gel under denaturing conditions.

These results are consistent with the expected change of molecular weight for the phosphorylated form of the KARAP at 12 kDa, observed by internal iodination. Furthermore, the immunoprecipitates of anti-CD158 mAbs prepared from KAR^+ NK cells comprise two other phospho-KARAPs migrating to 16 ± 1 kDa and 12 ± 1 kDa respectively (indicated by an asterisk on either side of the KARAP arrow at 14 kDa in Figure 3A).

Association of the KARs with a similar group of phosphorylated KARAPs was also observed with a panel of clones of KAR^+ NK cells and was absent from KIR^+ NK clones. It was seen that the relative intensity of the phospho-KARAPs at 16, 14 and 12 kDa can vary according to the origin of the NK cells.

Analysis of the phosphorylated amino acids revealed that the major KARAP at 14 kDa is principally phosphorylated on the tyrosine residues.

The results are illustrated in Figure 3C: the bands of KARAPs (on the left) and CD3ζ (on the right) were cut out after the *in vitro* kinase test and subjected to an analysis of the phosphorylated amino acids by thin layer electrophoresis. In this experiment, the KARAP and CD3ζ bands were isolated from immunoprecipitates of monoclonal antibodies, respectively anti-CD158 and anti-CD16 monoclonal antibodies, prepared from lyzates of R.P. NK cells.

Nevertheless, phosphorylation on the serine residues but not on the threonine residues can also be detected. As a control, analysis of the phosphorylated amino acids confirmed the phosphorylation of CD3ζ on the tyrosine residue only.

KARAPs and transduction of the activatory signal (KAR^+ transfectants)

By contrast with the p58.2 KIRs, the expression of p50.2 KAR in the transfectants of the RBL-2H3 non-lymphoid cell line does not lead to

reconstitution of the activatory function of the p50.2 KARs. In fact, the stimulation of transfectants of $p50.2^+$ RBL-2H3 cells induced by anti-CD158 antibodies does not lead to any detectable mobilization of the intracytoplasmic Ca^{2+} or to any detectable release of serotonin.

Remarkably, *in vitro* kinase tests carried out on the immunoprecipitates of anti-CD158 monoclonal antibodies prepared from transfectants of $p50.2^+$ RBL-2H3 cells did not include any detectable KARAP.

The results are illustrated in Figure 3B: lyzates prepared from $p50.2^+$ RBL-2H3 cells were immunoprecipitated with the indicated antibody (anti-CD3ε in lane 1, anti-FcεRIα in lane 2, anti-CD158 in lane 3) prior to *in vitro* kinase tests. The phosphorylated proteins were separated by SDS-PAGE on a 15% gel under denaturing conditions.

The lack of association of the KARs with the KARAPs in the transfectants of $p50.2^+$ RBL-2H3 cells was also confirmed by internal iodination (data not shown).

The KARAPs therefore associate selectively with the KARs and the absence of association of the KARs with the KARAPs is correlated with the inability of the KARs to transduce any detectable activatory signal.

Association of KARs with KARAPs (diagonal gel)

Finally, analysis of the immunoprecipitates of anti-CD158 monoclonal antibodies on a diagonal two-dimensional gel revealed that the phospho-KARAPs at about 16, 14 and 12 kDa decrease along the diagonal gel.

The results are illustrated in Figure 4: immunoprecipitates (IPs) of anti-CD158 monoclonal antibodies prepared from lyzates of R.P. NK cells were subjected to an *in vitro* kinase test prior to analysis by SDS-PAGE on a two-dimensional 13% gel under non-denaturing (horizontal direction)/denaturing (vertical direction) conditions.

These results thus indicate that the KARs are associated in the NK cells with a complex of KARAP dimers bonded by a disulfide linkage.

3. Discussion

KARs, which are autonomous activatory receptors, especially for class I MHC molecules, or coreceptors for the TCR (T cell receptor) or the cFR (immunoglobulin constant fragment receptor), represent a new way of activating the NK and T cells.

The inventors have shown that KARs are in fact assembled in the NK cells in the form of a multimeric complex involving KARAPs associated to form dimers bonded by a disulfide linkage.

Although analysis by radioiodination revealed one KARAP at about 12 ± 1 kDa, analysis by the kinase test revealed three phospho-KARAPs at about 16, 14 and 12 ± 1 kDa.

The correlation between the association of the KARs with the KARAPs and the activatory function of the KARs suggests that the KARAPs act as transducing subunits of the multimeric KAR complex.

However, the absence of association of the KARs with the KARAPs, as observed for the transfectants of RBL-2H3 cells, does not prevent expression of the receptor on the cell surface, contrary to what was observed in the case of the multimeric activatory receptors for antigens or antibodies including polypeptides with an ITAM (immunoreceptor activatory moiety based on tyrosine residue(s)).

Other activatory or at least non-inhibitory receptors of the immunoglobulin superfamily possess striking similarities to the p50 KARs (immunoglobulin-type human KARs): NKG2C/D lectin-type human KARs, pirA and gp49A immunoglobulin-type murine KARs, Ly49D and Ly49H lectin-type murine KARs, but also human activatory receptors of the LIR/MIR/ILT family, such as ILT1.

These similarities are illustrated in Figure 5, which shows the activatory or non-inhibitory receptors of the immunoglobulin superfamily (IgSF) or of the lectin type, and their inhibitory counterparts. Indicated underneath the name of each pair of receptors (from left to right: mPIR-B-mPIR-A, ILT2-ILT1, SIRPα-SIRPβ, KIR-KAR, FcγRIIB-FcγRIII, NKG2A/B-NKG2C/D, mLy49A/B/C/E/F/G/I-mLy49D/H) are the cells which express them naturally. The activatory or non-inhibitory receptors possess neither an ITIM (immunoreceptor inhibitory moiety based on tyrosine residue(s)) nor an ITAM (immunoreceptor activatory moiety based on tyrosine residue(s)), but do possess a charged amino acid residue in their transmembrane domain (TM) (R = arginine, K = lysine, D = aspartic acid, E = glutamic acid). The inhibitory counterparts (left item of each pair) contain an ITIM in their intracytoplasmic part (IC). In the extracytoplasmic part (EC), each activatory or non-inhibitory receptor has a high homology with its inhibitory counterpart.

EXAMPLE 2:

Biochemical characterization of the KARAP molecules (cf. Example 1 above) enabled us to specify the main identification criteria for the KARAP polypeptides, which were particularly as follows:

- polypeptides containing an extracytoplasmic cysteine amino acid allowing the formation of disulfide bridges (cf. Figure 4),
- polypeptides with an apparent molecular weight of between about 12 and 16 kDa, and
- polypeptides having at least one phosphorylatable tyrosine amino acid (cf. Figure 3C).

Given the strong similarities existing between the KARAP molecules identified at 12, 14 and 16 kDa, we assumed that these three molecular forms represented different degrees of phosphorylation of the same KARAP polypeptide, whose molecular weight could not exceed 12 kDa.

Furthermore, a major characteristic of KARAPs lies in their selective association with KARs and not with KIRs. Given that, in contrast to KIRs, KARs possess a transmembrane charged amino acid (lysine: K) and that this particular feature is also the basis of the association of the ITAM polypeptides present in the complexes CD3/TCR, BCR, FcεRI and FcγRIIIA (CD16), we orientated our strategy for identification of the KARAP gene by considering that KARAP is a new member of the family of the ITAM transmembrane polypeptides. In fact, the latter share the same characteristics with KARAP:

- polypeptides containing an extracytoplasmic cysteine amino acid (C) allowing the formation of disulfide bridges,
- polypeptides with a low molecular weight not exceeding 25 kDa,
- polypeptides having at least one phosphorylatable tyrosine amino acid included in an ITAM: YxxL/Ix_{6-8}YxxL/I, and
- presence of a transmembrane charged amino acid.

We thus developed a biological data processing strategy for identifying a gene from the public cDNA libraries available in the EST, GENBANK, SWISSPROT and EMBL forms. We used 2 different approaches:

1/ We translated all the ESTs according to the 6 reading frames, singling out only the peptides which had between 50 and 200 amino acids (envisaged molecular weight of between 5.5 and 22 kDa). We applied several selection criteria to this sub-base:

- Existence of a predicted transmembrane region of at least 10 amino acids, starting with amino acid 30, by Argos' method (Rao & Argos, 1986, Biochem. Biophys. Acta, 869, 197-214). In fact, by homology with the ITAM polypeptides

such as CD3ζ and FCεRIγ, the major part of the KARAP sequence is predicted as being intracytoplasmic.

- Search for an ITAM (Y-x-x-[IL]-x(6,8)-Y-x-x[IL]) in the C-terminal position of the transmembrane zone.

- Presence of a charged amino acid (R, K, D, E) in the transmembrane region.

- Presence of a cysteine amino acid (C) in the C-terminal position of the transmembrane zone.

2/ We searched for the EST entries (analysis also performed with EMBL, GENBANK and SWISSPROT) which had sequence similarities to the entry CD3Z_HUMAN. The program used was TBLASTN (version 1.4.11; Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers and David J. Lipman, 1990, J. Mol. Biol., 215, 403-10) or TBLASTN (version 2.0.3; Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller and David J. Lipman, 1997, Nucleic Acids Res., 25, 3389-3402). To these similar entries we then applied the selection criteria used in the first approach.

By combining these two biological data processing approaches and after having successively determined the leader, transmembrane, intracytoplasmic and extracytoplasmic regions of the candidate molecules with the aid of hydrophobicity profiles (Genworks and DNA Strider programs), we obtained a large number of sequences potentially corresponding to that of KARAP. Among these sequences, the one corresponding to accession number AA242315 in Genbank appeared to us to be the sequence of the murine KARAP gene (SEQ ID no. 1, C57Bl/6 murine cDNA). Figure no. 7 shows the DNA sequence (SEQ ID no. 1, cDNA) of a KARAP polypeptide according to the invention; this sequence corresponds to the sequence of the murine KARAP gene. In fact, translation of the nucleotide sequence gives an open reading frame of 396 nucleotides (SEQ ID no. 2). This result is illustrated in Figure no. 8, which shows that part of the nucleotide sequence of the KARAP gene (SEQ ID no. 1) which is between the leader sequence (excluded) and the stop codon, and which also shows, underneath this nucleotide sequence, the corresponding amino acid sequence (1-letter code) (SEQ ID no. 2, 3-letter code), i.e. the amino acid sequence of the mature murine KARAP according to the invention (SEQ ID no. 2). Standard analysis of this sequence predicts a mature protein of 87 amino acids (molecular weight of 9.6 kDa), an extracytoplasmic part of 16 amino acids (Q_1-G_{16}), a transmembrane part of 24 amino acids (V_{17}-G_{40}) and an intracytoplasmic part of 47 amino acids (R_{41}-R_{87}). According to our search strategy, the extracytoplasmic part comprises at least one cysteine amino acid (in fact two, C_8 and C_{10}), a transmembrane amino

acid (D_{25}) and an intracytoplasmic ITAM (Y_{65}QELQGQRHEVY$_{76}$SDL). Figure 9 illustrates the comparisons which can be made by aligning sequences between the ITAM polypeptides described previously and the polypeptide according to the invention possessing one (or more) ITAMs, and indicates the resulting consensus ITAM sequence: Figure 9 shows the alignment of the ITAMs of ITAM polypeptides (six CD3, one Igα, one Igβ, FcεRIγ and FcεRIβ) and an ITAM of the murine KARAP polypeptide (SEQ ID no. 2) identified above according to the invention (labelled "KARAP" in said Figure 9). On the basis of this comparison with the ITAMs described previously (Figure 9), we were able to envisage the association of the phosphorylated KARAPs with tyrosine kinase proteins containing SH2 groups in tandem (proteins such as ZAP-70 and p72Syk). The association of KARAPs with recombinant fusion proteins corresponding to the SH2 groups of ZAP-70 (preparation described in: Olcese L., Lang P., Vély F., Cambiaggi A., Marguet D., Bléry M., Hippen K.L., Biassoni R., Moretta A., Moretta L., Cambier J.C., Vivier E., 1996, J. Immunol., 156, 4531-4534) was verified *in vitro*: these experiments were carried out as described in Figure 3A, lane 3, but the cell lyzates were adsorbed by the recombinant fusion protein corresponding to the SH2 groups of ZAP-70 instead of the anti-CD158 antibody. Thus KARAP is a novel ITAM transmembrane molecule which associates with KARs and which, in a phosphorylated tyrosine form, associates with ZAP-70. KARAP is therefore a novel transducing element of T and NK lymphocytes. It is possible that KARAP or KARAP analogues also associate with the activatory isoforms of ITIM receptors and serve in these multimolecular complexes as subunits for transducing the signals emitted when the receptor is taken up.

A particularly appropriate method of determining or checking that a candidate polypeptide of known sequence corresponds to a KARAP according to the invention consists in producing an antibody against a characteristic part of this candidate polypeptide (for example an intracytoplasmic region comprising at least one ITAM, or an extracytoplasmic region) and in verifying that this antibody recognizes, on a functional cell, for example a functional KAR$^+$ cell, a target which is associated with the receptor for which the candidate polypeptide is assumed to be the KARAP (i.e., in the case of KAR$^+$ cells, verifying that the antibody recognizes a target which is associated with a KAR).

This method of identifying KARAP polypeptides according to the invention thus consists in particular in:

- producing a monoclonal or polyclonal antibody directed against this candidate polypeptide and in particular against a region of this candidate polypeptide which comprises at least one ITAM (for example, in the case of the murine KARAP identified above, an antibody directed against a region of the

extracytoplasmic part (SEQ ID no. 3) or the intracytoplasmic part (SEQ ID no. 5) of SEQ ID no. 2),

- bringing this antibody into contact with a lyzate of cells possessing, in a functional form, the activatory or non-inhibitory receptor for which the candidate polypeptide is assumed to constitute the KARAP, for example functional KAR^+ cells such as NK or T cells, under mild conditions allowing binding reactions of the antigen-antibody type, and

- identifying the candidate polypeptide as being a KARAP polypeptide according to the invention when the reaction products which may be formed contain a product whose apparent molecular weight is similar to that of a KAR (about 50 kDa) and a product whose apparent molecular weight is similar to that of the candidate polypeptide (especially between about 10 and 16 kDa).

This identification method according to the invention can be carried out in particular by:

- bringing said antibody into contact as described above,

- precipitating the reaction products which may be formed, under mild detergent conditions which preserve the molecular complexes (for example 1% digitonin; cf. Example 1 above),

- measuring the molecular weight of the precipitated products, for example by electrophoretic migration in the presence of molecular weight markers on a polyacrylamide gel under denaturing conditions, and

- identifying the candidate polypeptide as being a KARAP polypeptide according to the invention as described above.

EXAMPLE 3:

1° Identification of several ESTs corresponding to KARAP

Our strategy for cloning murine KARAP by biological data processing, as shown in Example 2 above, also reveals the existence of 5 ESTs (Expressed Tag Sequences) which correspond to our definition of KARAP. These are EST AA242315, AA734769, W88159, AA098506 and W41142. Figures 10A to 14A illustrate the cDNA sequences of EST AA242315, AA734769, W88159, AA098506 and W41142 respectively (SEQ ID no. 6 to SEQ ID no. 10 respectively). Figures 10B to 14B illustrate the protein sequences corresponding respectively to these ESTs (SEQ ID no. 11 to SEQ ID no. 15 for the proteins of EST AA242315, AA734769, W88159, AA098506 and W41142 respectively). All these ESTs were obtained from tissues taken from C57Bl/6 mice and were aligned in order to obtain a cDNA sequence corresponding to an open reading frame. This

is illustrated in Figure 15, which shows the alignment of the sequences of EST AA098506 (SEQ ID no. 9), AA242315 (SEQ ID no. 6), W88159 (SEQ ID no. 8), AA734769 (SEQ ID no. 7) and W41142 (SEQ ID no. 10) and shows the resulting consensus sequence (consensus murine KARAP cDNA; SEQ ID no. 16).

This is also illustrated in Figure 16, which shows the alignment of the protein sequences of EST AA242315 (SEQ ID no. 11), W88159 (SEQ ID no. 13), W41142 (SEQ ID no. 15), AA098506 (SEQ ID no. 14) and AA734769 (SEQ ID no. 12) and shows the resulting consensus sequence (consensus murine KARAP; SEQ ID no. 17). In these Figures 15 and 16, the symbol "." indicates an identity with the consensus sequence in question and the symbol "-" indicates the absence of sequencing data.

2° Genomic sequence of murine KARAP

A library of genomic DNA (phage lambda, EMBL3), isolated from mice of the 129 murine line, was screened with the cDNA corresponding to the sequence of EST AA734769 by a conventional technique. A phage containing an 18 kb fragment was identified as positive. This phage was mapped by cleavage with a series of restriction enzymes and a 9 kb EcoRI-EcoRI fragment obtained from the phage was cloned into cloning vector pBlue-Script and contains the whole of the murine KARAP gene (from the initial ATG to the STOP sequence). The sequence of this murine KARAP gene is shown in Figure 17 (SEQ ID no. 18; 2838 bp).

Furthermore, oligonucleotide primers were generated in order to obtain the genomic organization of murine KARAP. The primers used are shown in Table 1 below (SEQ ID no. 19 to SEQ ID no. 26):

Table I

Identification no.	Strandedness	Position*	Sequence (5'-3')	
7134	Sense	60-81	GGC TCT GGA GCC CTC CTG GTG C	SEQ ID No. 19
7132	Antisense	581-561	ACT CTG GGC CTG TAC GGG ACT	SEQ ID No. 20
7133	Sense	561-581	AGT CCC GTA CAG GCC CAG AGT	SEQ ID No. 21
7130	Antisense	800-780	CAG AGT CAA CAC CAA GTC ACC	SEQ ID No. 22
7131	Sense	780-800	GGT GAC TTG GTG TTG ACT CTG	SEQ ID No. 23
7128	Antisense	978-958	CTC AGT CTC AGC AAT GTG TTG	SEQ ID No. 24
7129	Sense	958-978	CAA CAC ATT GCT GAG ACT GAG	SEQ ID No. 25
7127	Antisense	2703-2683	CTG TGT GTT GAG GTC ACT GTA	SEQ ID No. 26

* Position according to the genomic sequence

The genomic organization of murine KARAP is shown in Figure 18. We also obtained the cDNA sequence and hence the protein sequence of murine KARAP of the 129 line from these data. This cDNA sequence (SEQ ID no. 27) and this protein sequence (SEQ ID no. 28) are shown in Figure 19.

The protein sequence translated in this way is:

MGALEPSWCLLFLPVLLTVLGLSPVQA	Signal sequence
QSDTFPRCDCSSVPG	Extracytoplasmic domain
VLAGIVLGDLVLTLLIALAYSLG	Transmembrane domain
RLVSRGQERTRKQHIAETESPYQELQGQRPEVYSDLNTQRQYYR	Intracytoplasmic domain

Considered together, these genomic mapping results show that the murine KARAP gene (from the initial ATG to the STOP sequence) has a length of about 2.9 kb and comprises 5 exons. These results are illustrated in Figure 20, which shows, from top to bottom, the genomic DNA of murine KARAP of 129 mice (black: translated exon; horizontal hatching: untranslated exon; white: intron), the corresponding protein sequence (SEQ ID no. 28 from exon no. 1 to exon no. 5) and the nature of the different regions of this protein (SS = signal sequence; EC = extracytoplasmic domain; TM = transmembrane domain; IC = intracytoplasmic domain). Exon 1 codes for an N-terminal portion of the signal sequence, exon 2 codes for the remainder of the signal sequence and the first three amino acids of the extracytoplasmic part, exon 3 codes for the remainder of the extracytoplasmic part, the transmembrane part and the first 9 amino acids of the intracytoplasmic part, exon 4 codes for the 14 amino acids of the intracytoplasmic part and exon 5 codes for the remainder of the protein. As expected for the genomic organization of an ITAM polypeptide like KARAP, the ITAM is coded for by two exons (exons 4 and 5) separated by a type 0 intron.

3° Functional reconstitution of a KAR (p50.2) expressed in RBL-2H3 cells by the human KARAP DAP-12

We obtained the cDNA coding for human KARAP by RT-PCR, generating oligonucleotide primers deduced from the sequence of murine KARAP. The primers used are shown in Table 2 below (SEQ ID no. 29 and no. 30):

Table 2

Identification no.	Strandedness	Location*	Sequence (5'-3')
7367	Sense	ATG (53)	CCGCTCGAGGGCTTCATGGGGGGACTTGAAC (SEQ ID No. 29)
			Xho I Start codon
7368	Antisense	398	CTAGTCTAGA GGATCCAGGTATCATTGTGCTGACTGTCATGATTCG(398)
			Xba I Bam III (SEQ ID No. 30)

* Numbering as a function of the murine cDNA sequence (SEQ ID No. 27)

The sequence of the cDNA obtained is shown in Figure 21 (SEQ ID no. 31; cDNA of human KARAP). RNA extracted from KAR^+ human NK clones was used as the base for generating this cDNA. This cDNA was cloned into eukaryotic expression vector pNT-neo and stable transfectants for this human KARAP were generated in the KAR^+ transfectant (p50.2) of the RBL-2H3 cell line (Bléry et al., J. Biol. Chem., 1997). The capacity of the KARs expressed on the now doubly transfected $p50.2^+$ and $KARAP^+$ RBL-2H3 cells to transduce an activatory signal was tested by stimulation with antibodies directed against the extracytoplasmic part of p50.2 and by following the release of tritiated serotonin.

The protocol adopted for this tritiated serotonin release experiment is as follows:

These cells are detached, centrifuged and resuspended in RPMI/10% FCS at a final concentration of 1×10^6 cells per ml. The cells are then incubated for 1 hour with 2 μCi of tritium-labelled serotonin per ml of cells. The cells are washed and then reintroduced into medium for 1 hour at 37°C so that they release the excess serotonin from their stocks. The cells are then distributed into 96-well plates (200,000 cells per well) with mouse IgE (2682-I) or an anti-p50 Ab (GL183). The cells adhere for 1 hour and are then washed. They are returned to 37°C for 15 minutes and then stimulated with F(ab')2 GAM (50 μg/ml). The cells are left for 30 minutes at 37°C to enable them to release their serotonin. The reaction is stopped by adding cold HBSS and placing the cells on ice. Half of the supernatant from each well is then recovered and placed in 1 ml of scintillation liquid. 100% degranulation is obtained from the same volume of lyzate obtained from cells which incorporate serotonin but are not stimulated. The samples are then counted on a β counter.

The results obtained are illustrated in Figure 22, which shows the % of serotonin released into the supernatant by the doubly transfected p50/human KARAP RBL-2H3 cells stimulated by the antibody indicated on the abscissa (left: no antibody; centre: mouse IgE : mIgE 1/500; right: GL183 5 μg/ml). As indicated in this Figure 22, whereas the uptake of KAR into RBL-2H3 by an antibody reacting with the extracytoplasmic part of KAR (the monoclonal antibody GL183) does not result in activation of the cells, the uptake of KAR by GL183 in the RBL-2H3 double transfectants expressing both human KAR and human KARAP does result in cell activation (objectified here by the release of serotonin from the cells). This is therefore the formal proof that the identified human KARAP sequence reconstitutes the functionality of KARs.

Figure 23 illustrates the homology between the organization of the human KARAP gene and that of the murine KARAP gene (E1 to E5: exon 1 to exon 5; I1

to I4: intron 1 to intron 4). The numbering of the base pairs of the human and murine KARAP genes is indicated in said Figure.

CLAIMS

1. Isolated polypeptide, characterized in that it makes it possible to restore a deficient KAR activation, or a fragment or homologue of such a polypeptide.

2. Polypeptide according to claim 1, characterized in that it is capable of associating with a KAR and not associating with the inhibitory counterpart of this KAR, or a fragment or homologue of such a polypeptide.

3. Polypeptide as obtained:

i. by immunoprecipitation of one or more polypeptide fractions of lysates of cells expressing KAR receptors capable of transducing an activating signal, with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

ii. it optionally being possible for each polypeptide fraction to be exhausted beforehand by removal of the fractions immunoprecipitated with anti-CD3 and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1, anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

iii. by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight, and recovery of the polypeptides corresponding to a molecular weight of about 12 ± 2 kDa, or

by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight after said polypeptide fraction(s) has (have) been subjected to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of about 12, 14 and/or 16 ± 2 kDa,
or a fragment or homologue of such a polypeptide.

4. Polypeptide according to claim 3, characterized in that said cells are NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes.

5. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence:

- has at least one phosphorylatable tyrosine amino acid, and
- has a molecular weight of between about 10 ± 2 and 16 ± 2 kDa.

6. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence contains at least one ITAM YxxL/Ix_{6-8}YxxL/I.

7. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence contains an extracytoplasmic region, a transmembrane region and an intracytoplasmic region.

8. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence contains at least one extracytoplasmic cysteine amino acid.

9. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence contains at least one transmembrane charged amino acid (R, K, D, E).

10. Polypeptide according to any one of the preceding claims, characterized in that it is phosphorylated on at least one tyrosine residue.

11. Polypeptide according to any one of the preceding claims, characterized in that it is in the form of dimers.

12. Polypeptide according to any one of the preceding claims, characterized in that it binds to a molecule having an SH2 or PTB domain.

13. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence essentially consists of SEQ ID no. 2, no. 3, no. 4, no. 5, no. 11, no. 12, no. 13, no. 14, no. 15, no. 17 or no. 28.

14. Polypeptide according to any one of the preceding claims, characterized in that it is modified by glycosylation, phosphorylation, sulphonation, biotinylation, acylation or esterification, by the addition, substitution or suppression of entities whose molecular shape is similar to that of phosphate groups, such as phosphonate, by the addition of tracer reagents such as luciferase, GFP (*Green Fluorescence Protein*) or analogues thereof, by the addition of purification targets such as an affinity ligand, or by the addition of entities modifying its solubility.

15. Polypeptide according to any one of the preceding claims, characterized in that it is capable of crossing a cell membrane.

16. Polypeptide according to any one of the preceding claims, characterized in that it is modified so as to inhibit its capacity to transduce a signal.

17. Polypeptide according to claim 17, characterized in that it is modified so as to be non-hydrolyzable under biological conditions, especially by the addition of phosphonate groups.

18. Polypeptide according to claim 17, characterized in that it is modified by substitution of a tyrosine residue with a phenylalanine residue.

19. Antibody or fragment of such an antibody, particularly an Fc, Fv, Fab, F(ab)'2 or CDR fragment, as obtained by immunogenesis from a polypeptide according to any one of the preceding claims or from a fragment of such a polypeptide.

20. Antibody or antibody fragment according to claim 20, characterized in that it is capable of recognizing SEQ ID no. 2, SEQ ID no. 3, SEQ ID no. 4, SEQ ID no. 5, SEQ ID no. 11, SEQ ID no. 12, SEQ ID no. 13, SEQ ID no. 14, SEQ ID no. 15, SEQ ID no. 17 and/or SEQ ID no. 28.

21. Nucleic acid or variant of such a nucleic acid, characterized in that it comprises a sequence corresponding to the open reading frame, according to the universal genetic code, of the amino acid sequence of a polypeptide according to any one of the preceding claims.

22. Nucleic acid according to claim 22, or variant of such a nucleic acid, characterized in that said nucleic acid has a sequence essentially consisting of SEQ ID no. 2, no. 6, no. 7, no. 8, no. 9, no. 10, no. 16, no. 27, no. 31 or no. 18.

23. Process for obtaining a polypeptide according to any one of the preceding claims, characterized in that it comprises steps involving:

i. immunoprecipitation of one or more polypeptide fractions of lysates of KAR^+ cells with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

ii. it optionally being possible for each polypeptide fraction to be exhausted beforehand by removal of the fractions immunoprecipitated with anti-CD3 and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140

antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody, and

iii. separation of the polypeptides of said polypeptide fraction(s) according to their molecular weight, and recovery of the polypeptides corresponding to a molecular weight of about 12 ± 2 kDa, or

separation of the polypeptides of said polypeptide fraction(s) according to their molecular weight after said polypeptide fraction(s) has (have) been subjected to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of about 12, 14 and/or 16 ± 2 kDa.

24. Process according to claim 23, characterized in that said KAR^+ cells are NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes.

25. Method of obtaining the sequence of a polypeptide according to any one of Claims 1 to 18, characterized in that it is carried out by screening candidate sequences so as to select only that (those) which:

- has (have) at least one phosphorylatable tyrosine amino acid,
- has (have) a molecular weight of between about 5 and 25 kDa,
- contains (contain) an extracytoplasmic region, a transmembrane region and an intracytoplasmic region,
- contains (contain) at least one cysteine amino acid in its extracytoplasmic region,
- contains (contain) at least one charged amino acid (R, K, D, E) in its transmembrane region, and
- contains (contain) at least one ITAM $YxxL/Ix_{6-8}YxxL/I$ in its intracytoplasmic region,

and in that it is ensured that the polypeptide(s) corresponding to the selected sequence(s) is (are) capable of associating with a KAR receptor while at the same time not associating with the counterpart receptor which inhibits this KAR.

26. Method of determining whether a candidate polypeptide corresponds to a polypeptide according to any one of Claims 1 to 18, characterized in that it comprises:

- producing a monoclonal or polyclonal antibody directed against this candidate polypeptide and in particular against an extracytoplasmic region of this candidate polypeptide and/or a region comprising at least one ITAM unit,
- bringing this antibody into contact with a lysate of cells possessing, in a functional form, the activating or non-inhibitory receptor for which the candidate

polypeptide is assumed to constitute the KARAP, under mild conditions allowing binding reactions of the antigen-antibody type,

- identifying the candidate polypeptide as being a polypeptide according to any one of Claims 1 to 18 when the reaction products which may be formed contain a product whose apparent molecular weight is similar to that of said activating or non-inhibitory receptor, and a product whose apparent molecular weight is similar to that of the candidate polypeptide.

27. Pharmaceutical composition comprising, in association with a pharmaceutically acceptable vehicle, an effective amount of polypeptides according to any one of the preceding claims, or fragments of such polypeptides, or an effective amount of antibodies according to claim 19 or 20, or fragments of such antibodies, or an effective amount of nucleic acids according to claim 21 or 22, or variants of such nucleic acids.

28. *In vitro* method of diagnosing an abnormal or undesired function of a cell, characterized in that it comprises steps involving:

- bringing of at least one cell, or one cell extract, into contact with an antibody according to claim 21 or 22, or a fragment of such an antibody, or with a nucleic acid according to claim 21 or 22, or a variant of such a nucleic acid, and
- revealing of the reaction product which may be formed.

29. *In vitro* diagnostic method according to claim 28, characterized in that said abnormal or undesired function results in an immunoproliferative disease, an immunodeficiency disease such as an HIV disease, a cancer such as lymphoproliferative disease of the granular lymphocytes, an autoimmune disease such as rheumatoid arthritis, an infectious disease such as malaria, an allergic response or a graft reject.

30. Method of identifying molecules which adapt or carry out the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with polypeptides according to any one of Claims 1 to 18 (or with fragments of such polypeptides), and

ii. selection of those candidate molecules for which a binding to said polypeptides (or to said polypeptide fragments) is observed.

31. Method of identifying molecules capable of modulating a cell activity resulting from the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with molecules which adapt or carry out the activation of a KAR, as obtained by the method according to claim 30, and with polypeptides according to any one of claims 1 to 18 (or with fragments of such polypeptides), and

ii. selection of those candidate molecules which exert an effect on the binding between said polypeptides (or said polypeptide fragments) and said adapter or effector molecules, as observed in the absence of said candidate molecules.

CLAIMS

1. Isolated polypeptide, characterized in that it makes it possible to restore a deficient KAR activation, in that it is capable of associating with a KAR, and not associating with the inhibitory counterpart of this KAR, and in that its amino acid sequence:

- has at least one phosphorylatable tyrosine amino acid,
- has a molecular weight comprised between approximately 10 + 2 and 16 + 2 kDa,
- contains at least one ITAM YxxL/Ix_{6-8}YxxL/I unit,
- contains an extracytoplasmic region, a transmembrane region and an intracytoplasmic region,
- contains at least one extracytoplasmic cysteine amino acid,
- contains at least one transmembrane charged amino acid (R, K, D, E),

or a fragment or homologue of such a polypeptide, said homologues or fragments being capable of transducing a signal originating from a KAR.

2. Polypeptide as obtained:

i. by immunoprecipitation of one or more polypeptide fractions of lysates of cells expressing KAR receptors capable of transducing an activating signal, with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

ii. it optionally being possible for each polypeptide fraction to be exhausted beforehand by removal of the fractions immunoprecipitated with anti-CD3 and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1, anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

iii. by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight, and recovery of the polypeptides corresponding to a molecular weight of about 12 ± 2 kDa, or

by resolution of the polypeptides of said polypeptide fraction(s) according to their molecular weight after said polypeptide fraction(s) has (have) been subjected to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of about 12, 14 and/or 16 ± 2 kDa,

or a fragment or homologue of such a polypeptide, said homologues or fragments being capable of transducing a signal originating from a KAR.

3. Polypeptide according to claim 1 or 2, characterized in that said cells are NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes.

4. Polypeptide according to any one of the preceding claims, characterized in that it is phosphorylated at the level of at least one tyrosine residue.

5. Polypeptide according to any one of the preceding claims, characterized in that it is in the form of dimers.

6. Polypeptide according to any one of the preceding claims, characterized in that it binds to a molecule having an SH2 or PTB domain.

7. Polypeptide according to any one of the preceding claims, characterized in that its amino acid sequence essentially consists of SEQ ID No. 2, no. 3, no. 4 or no. 5, no. 11, no. 12, no. 13, no. 14, no. 15, no. 17 or no. 28.

8. Polypeptide according to any one of the preceding claims, characterized in that it is modified by glycosylation, phosphorylation, sulphonation, biotinylation, acylation or esterification, by the addition, substitution or suppression of entities whose molecular shape is similar to that of phosphate groups, such as phosphonate, by the addition of tracer reagents such as luciferase, GFP (*Green Fluorescence Protein*) or analogues thereof, by the addition of purification targets such as an affinity ligand, by the addition of entities modifying its solubility.

9. Polypeptide according to any one of the preceding claims, characterized in that it is capable of crossing a cell membrane.

10. Polypeptide according to any one of the preceding claims, characterized in that it is modified so as to inhibit its capacity to transduce a signal.

11. Polypeptide according to claim 10, characterized in that it is modified so as to be non-hydrolyzable under biological conditions, especially by the addition of phosphonate groups.

12. Polypeptide according to claim 10, characterized in that it is modified by substitution of a tyrosine residue with a phenylalanine residue.

13. Antibody or fragment of such an antibody, particularly an Fc, Fv, Fab, F(ab)'2 or CDR fragment, as obtained by immunogenesis from a polypeptide according to any one of the preceding claims or from a fragment of such a polypeptide.

14. Antibody or antibody fragment according to claim 19, characterized in that it is capable of recognizing SEQ ID no. 2, SEQ ID no. 3, SEQ ID no. 4, SEQ ID no. 5, SEQ ID no. 11, SEQ ID no. 12, SEQ ID no. 13, SEQ ID no. 14, SEQ ID no. 15, SEQ ID no. 17 and/or SEQ ID no. 28.

15. Nucleic acid or variant of such a nucleic acid, characterized in that it comprises a sequence corresponding to the open reading frame, according to the universal genetic code, of the amino acid sequence of a polypeptide according to any one of the preceding claims.

16. Nucleic acid according to claim 15, or variant of such a nucleic acid, characterized in that said nucleic acid has a sequence essentially consisting of SEQ ID no. 1, no. 6, no. 7, no. 8, no. 9, no. 10, no. 16, no. 27, no. 31 or no. 18.

17. Method of obtaining a polypeptide according to any one of the preceding claims, characterized in that it comprises steps involving:

i. immunoprecipitation of one or more polypeptide fractions of lysates of KAR^+ cells with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody,

ii. it optionally being possible for each polypeptide fraction to be exhausted beforehand by removal of the fractions immunoprecipitated with anti-CD3 and/or anti-FcεRIγ antibodies, and/or to be reprecipitated with one or more anti-KIR and/or anti-KAR antibodies such as an anti-CD158, anti-p70/NKB1 or anti-p140 antibody and more particularly the EB6, GL183 or PAX250 monoclonal antibody, and

iii. separation of the polypeptides of said polypeptide fraction(s) according to their molecular weight, and recovery of the polypeptides corresponding to a molecular weight of about 12 ± 2 kDa, or

separation of the polypeptides of said polypeptide fraction(s) according to their molecular weight after said polypeptide fraction(s) has (have) been subjected to a kinase test, and recovery of the phosphorylated polypeptides corresponding to a molecular weight of about 12, 14 and/or 16 ± 2 kDa.

18. Method according to claim 17, characterized in that said KAR^+ cells are NK cells and/or T cells and/or myeloid cells and/or B cells and/or mastocytes.

19. Method of obtaining the sequence of a polypeptide according to any one of claims 1 to 12, characterized in that it is carried out by screening candidate sequences so as to select only that (those) which:

- has (have) at least one phosphorylatable tyrosine amino acid,
- has (have) a molecular weight of between about 5 and 25 kDa,
- contains (contain) an extracytoplasmic region, a transmembrane region and an intracytoplasmic region,
- contains (contain) at least one cysteine amino acid in its extracytoplasmic region,
- contains (contain) at least one charged amino acid (R, K, D, E) in its transmembrane region, and
- contains (contain) at least one ITAM $YxxL/Ix_{6-8}YxxL/I$ in its intracytoplasmic region,

and in that it is ensured that the polypeptide(s) corresponding to the selected sequence(s) is (are) capable of associating with a KAR receptor while at the same time not associating with the counterpart receptor which inhibits this KAR.

20. Method of determining whether a candidate polypeptide corresponds to a polypeptide according to any one of claims 1 to 12, characterized in that it comprises:

- producing a monoclonal or polyclonal antibody directed against this candidate polypeptide and in particular against an extracytoplasmic region of this candidate polypeptide and/or a region comprising at least one ITAM unit,
- bringing this antibody into contact with a lysate of cells possessing, in a functional form, the activatory or non-inhibitory receptor for which the candidate polypeptide is assumed to constitute the KARAP, under mild conditions allowing binding reactions of the antigen-antibody type,
- identifying the candidate polypeptide as being a polypeptide according to any one of claims 1 to 12 when the reaction products which may be formed contain a product whose apparent molecular weight is similar to that of said activatory or non-inhibitory receptor, and a product whose apparent molecular weight is similar to that of the candidate polypeptide.

21. Pharmaceutical composition comprising, in association with a pharmaceutically acceptable vehicle, an effective amount of polypeptides according to any one of the preceding claims, or fragments of such polypeptides,

or an effective amount of antibodies according to claim 13 or 14, or fragments of such antibodies, or an effective amount of nucleic acids according to claim 15 or 16, or variants of such nucleic acids.

22. *In vitro* method of diagnosing an abnormal or undesired function of a cell, characterized in that it comprises steps involving:

- bringing of at least one cell, or one cell extract, into contact with an antibody according to claim 13 or 14, or a fragment of such an antibody, or with a nucleic acid according to claim 15 or 16, or a variant of such a nucleic acid, and
- revealing of the reaction product which may be formed.

23. *In vitro* diagnostic method according to claim 22, characterized in that said abnormal or undesired function results in an immunoproliferative disease, an immunodeficiency disease such as an HIV disease, a cancer such as lymphoproliferative disease of the granular lymphocytes, an autoimmune disease such as rheumatoid arthritis, an infectious disease such as malaria, an allergic response or a graft reject.

24. Method of identifying molecules which adapt or carry out the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with polypeptides according to any one of claims 1 to 12 (or with fragments of such polypeptides), and

ii. selection of those candidate molecules for which a binding to said polypeptides (or to said polypeptide fragments) is observed.

25. Method of identifying molecules capable of modulating a cell activity resulting from the activation of a KAR, characterized in that it comprises steps involving:

i. bringing of the candidate molecules into contact with molecules which adapt or carry out the activation of a KAR, as obtained by the method according to claim 24, and with polypeptides according to any one of claims 1 to 12 (or with fragments of such polypeptides), and

ii. selection of those candidate molecules which exert an effect on the binding between said polypeptides (or said polypeptide fragments) and said adapter or effector molecules, as observed in the absence of said candidate molecules.

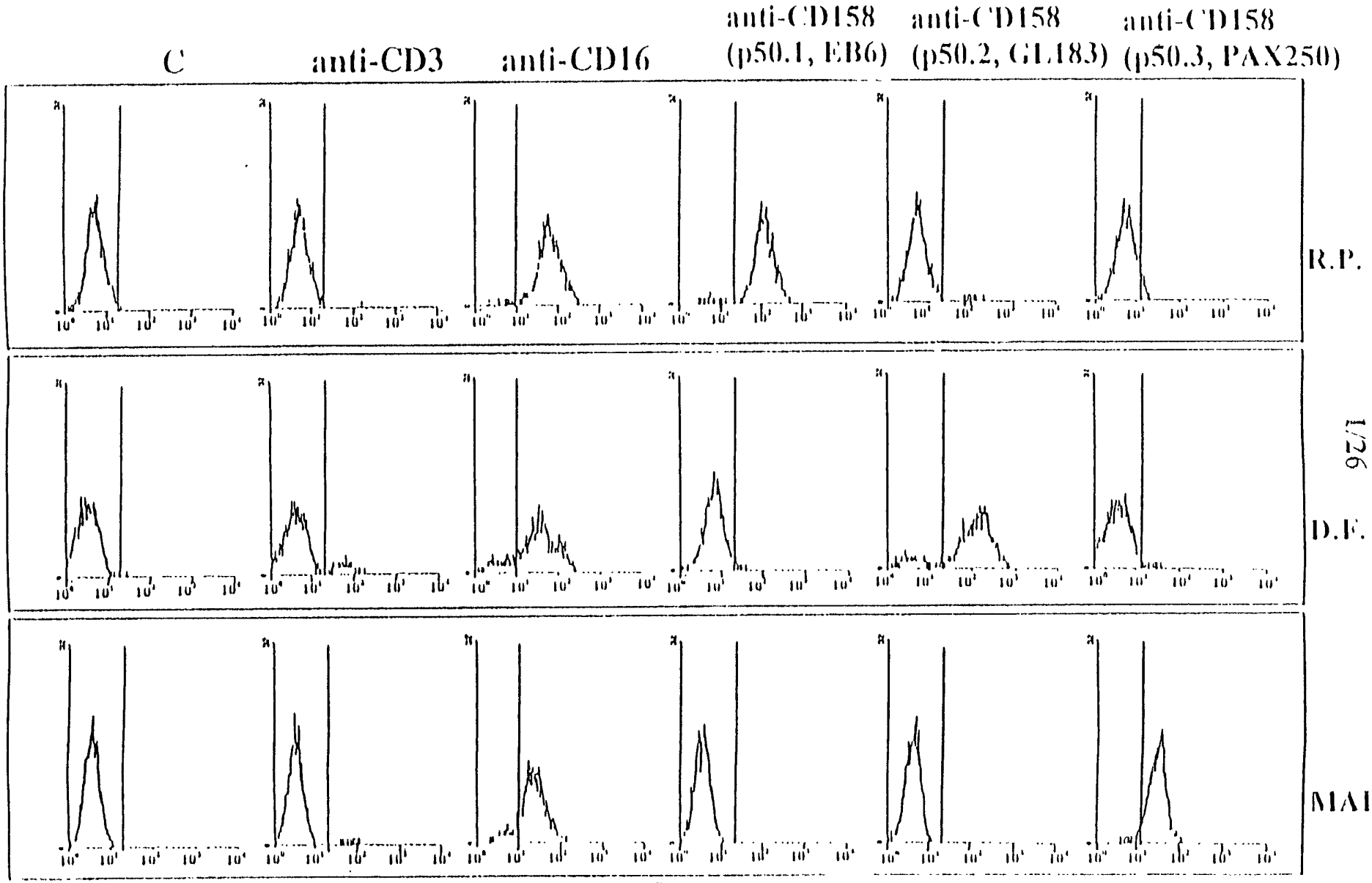


Figure 1A

Figure 1B

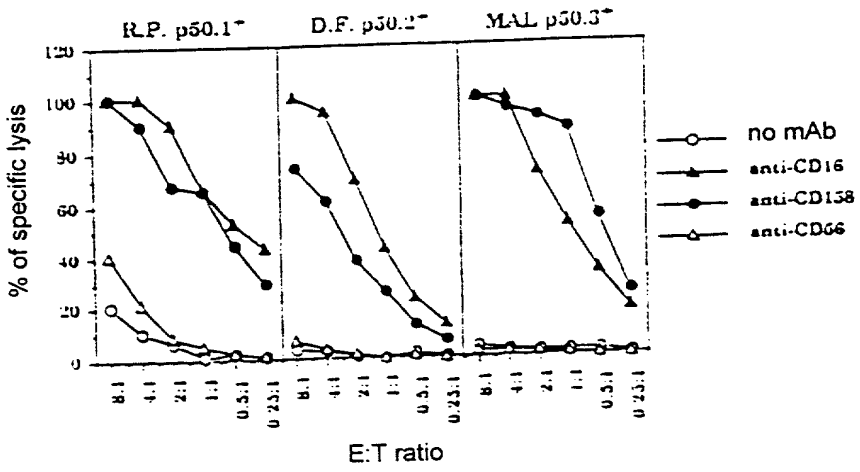

R.P. p50.1+
D.F. p50.2+
MAL p50.3+
% of specific lysis
E:T ratio
no mAb
anti-CD16
anti-CD158
anti-CD66

Figure 2A

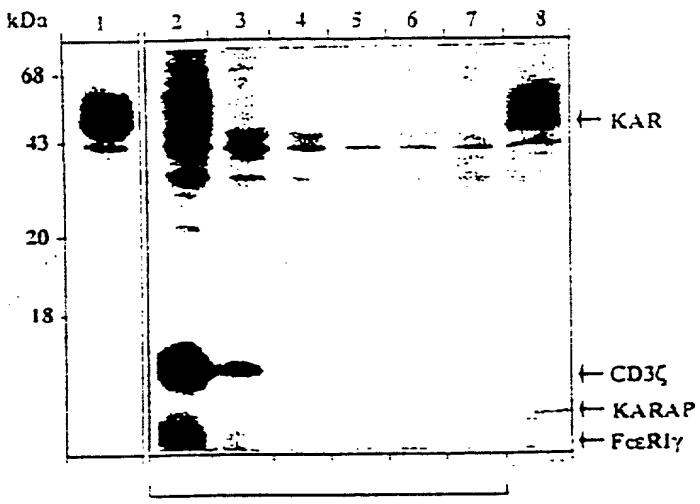


Figure 2B

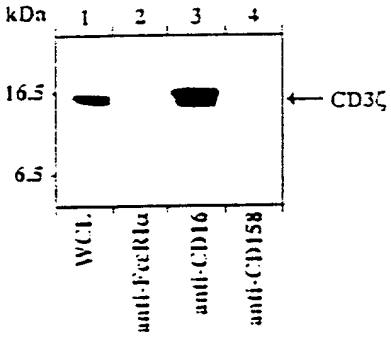


Figure 3A

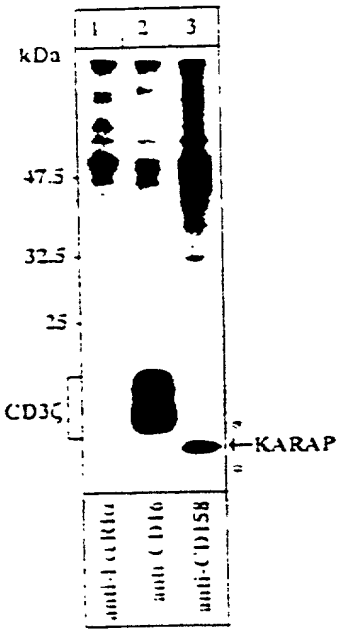


Figure 3B

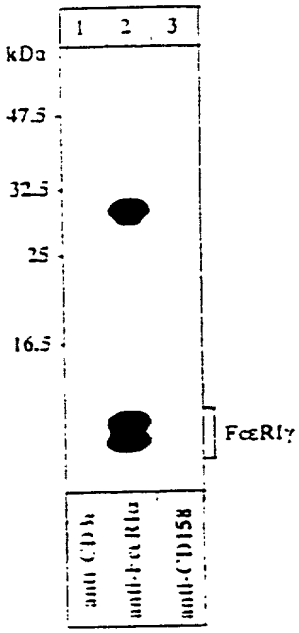


Figure 3C

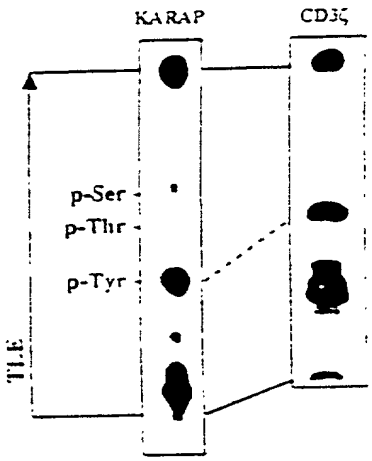
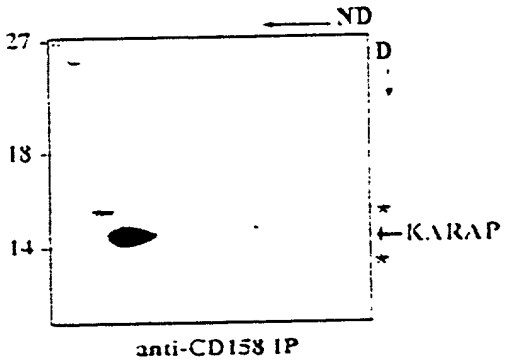


Figure 4



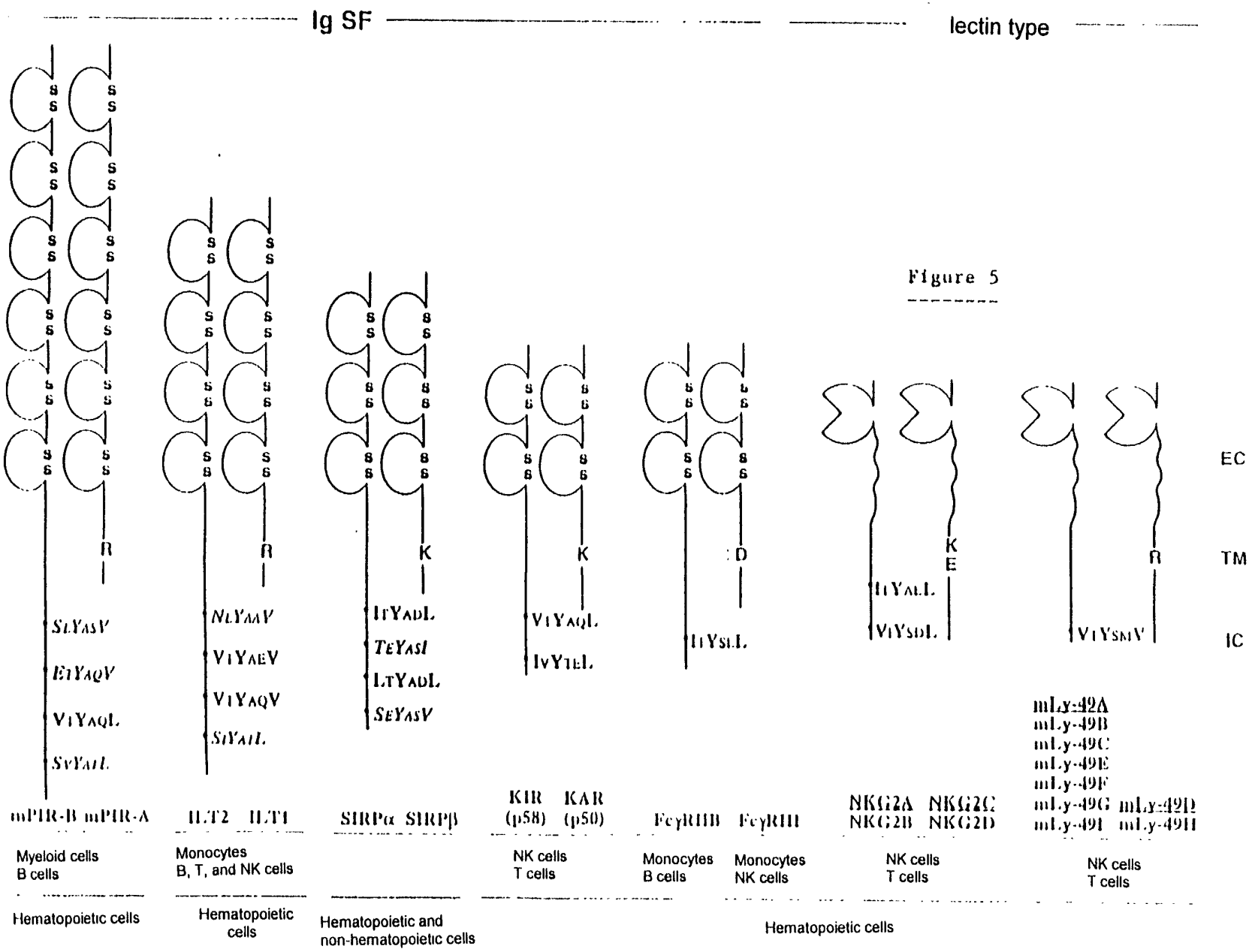


Figure 5

NK p58/50 cell receptors for
class I MHC molecules

Figure 6

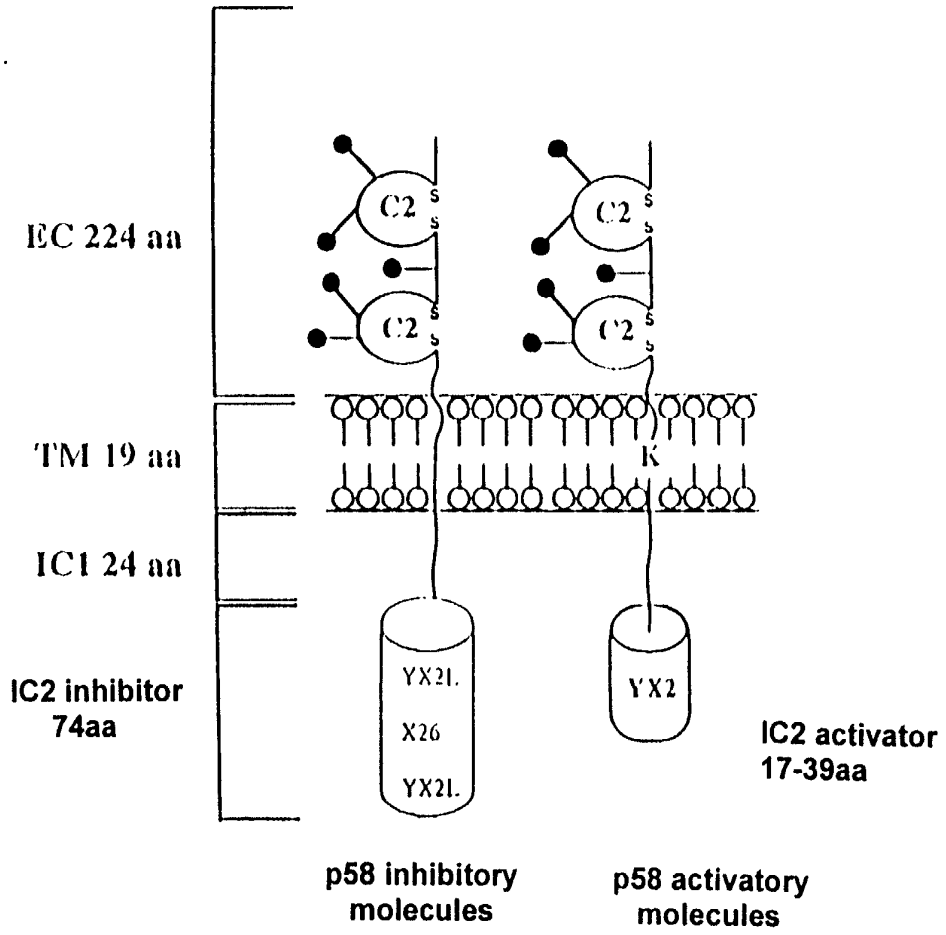


Figure 7

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  1 ggtcacacca ggtcccacca gccccggac tgtggtgtcc agtgcatatc tggccaccat
 61 ggggctctgg agcctcctgg tgccttctgt tcttcctgt cctcctgact gtgggaggat
121 taagtcccgt acaggcccag agtgacactt tcccaagatg cgactgttct tccgtgagcc
181 ctggtgtact gtctgggatt gttctgggtg acttggtgtt gactctgctg attgccctgg
241 ctgtgtactc tctgggccgc ctggtctccc gaggtcaagg gacagcggaa gggaccccga
301 aacaacacat tgctgagact gagtcgccct atcaggagct tcagggtcag agacatgaag
361 tatacagtga cctcaacaca cagaggccat attacagatg agcccactct atgcccatca
421 gcggcctgat gcccggatcc ggtcattcca gatgcctact caacaagccc tctctgaga
481 caggactccc gtggaatac agatcccacag ggtacct
```

Figure 8

```
1/1                                                 31/11
cag agt gac act ttc cca aga tgc gac tgt tct tcc gtg agc cct ggt gta ctg tct ggg
Q   S   D   T   F   P   R   C   D   C   S   S   V   S   P   G   V   L   S   G
61/21                                               91/31
att gtt ctg ggt gac ttg gtg ttg act ctg ctg att gcc ctg gct gtg tac tct ctg ggc
I   V   L   G   D   L   V   L   T   L   L   I   A   L   A   V   Y   S   L   G
121/41                                              151/51
cgc ctg gtc tcc cga ggt caa ggg aca gcg gaa ggg acc cgg aaa caa cac att gct gag
R   L   V   S   R   G   Q   G   T   A   E   G   T   R   K   Q   H   I   A   E
181/61                                              211/71
act gag tcg cct tat cag gag ctt cag ggt cag aga cat gaa gta tac agt gac ctc aac
T   E   S   P   Y   Q   E   L   Q   G   Q   R   H   E   V   Y   S   D   L   N
241/81
aca cag agg caa tat tac aga
T   Q   R   Q   Y   Y   R
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Figure 9

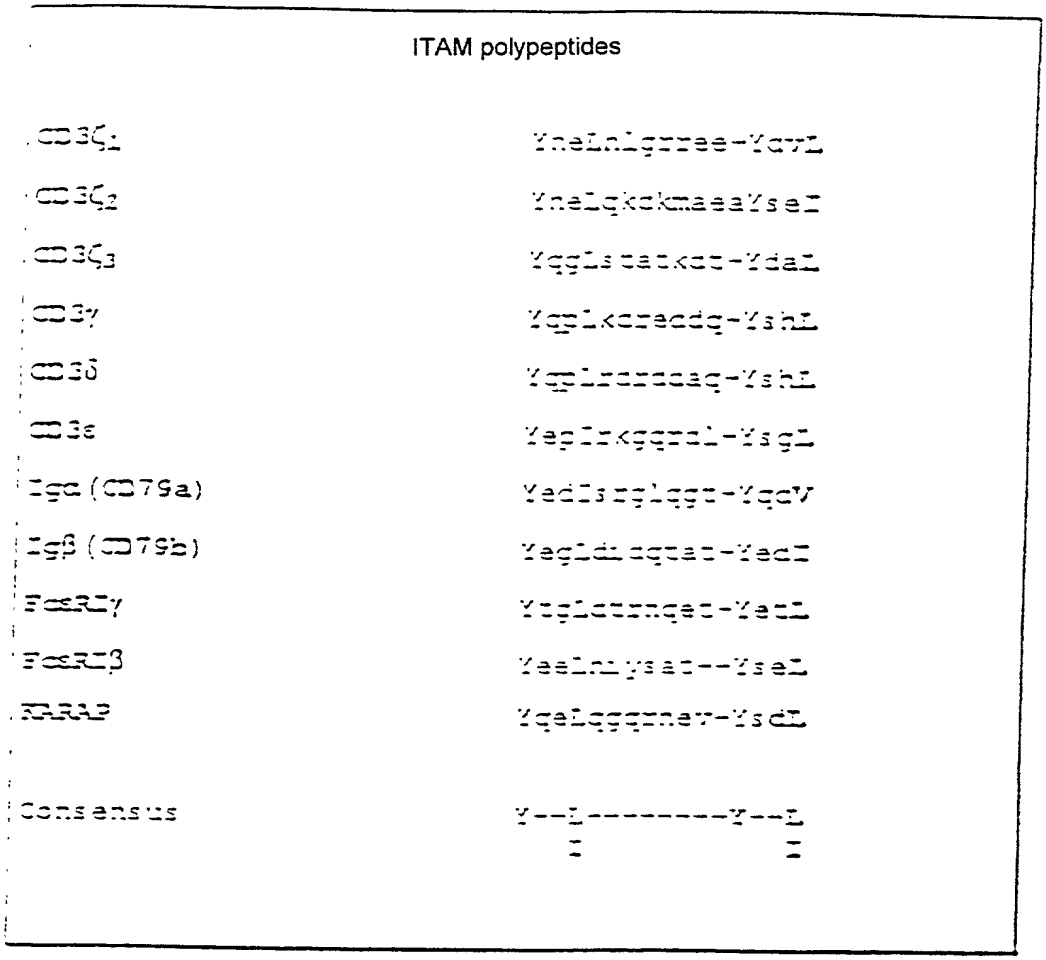
ITAM polypeptides
CD3ζ1 YneLnlgrree-YdvL
CD3ζ2 YneLqkdkmaeaYseI
CD3ζ3 YqgLstatkdt-YdaL
CD3γ YqpLkdneddq-YshL
CD3δ YqpLrdredaq-YshL
CD3ε YepLrkgqrdl-YsgL
Igα (CD79a) YedLstglqgt-YqdV
Igβ (CD79b) YeqLdLdqdat-YedI
FcεRIγ YtgLstnnqet-YetL
FcεRIβ YeeLnLysat--YseL
KARAP YqeLqgqrnev-YsdL
Consensus Y--L--------Y--L
I I

Figure 10A

SEQ ID n°6

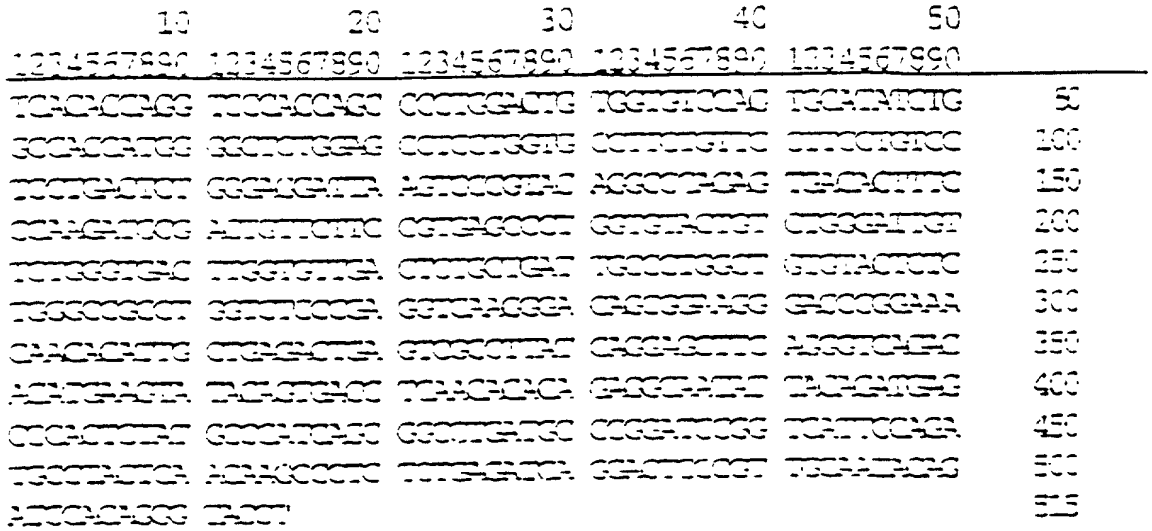

Figure 10B

SEQ ID n°11

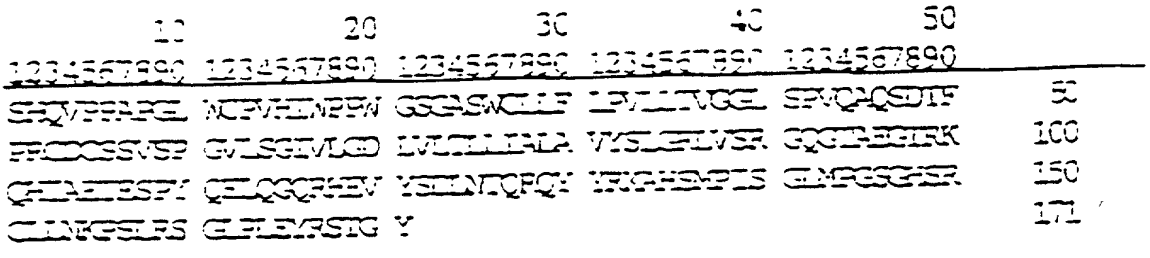

Figure 11A

SEQ ID n°7

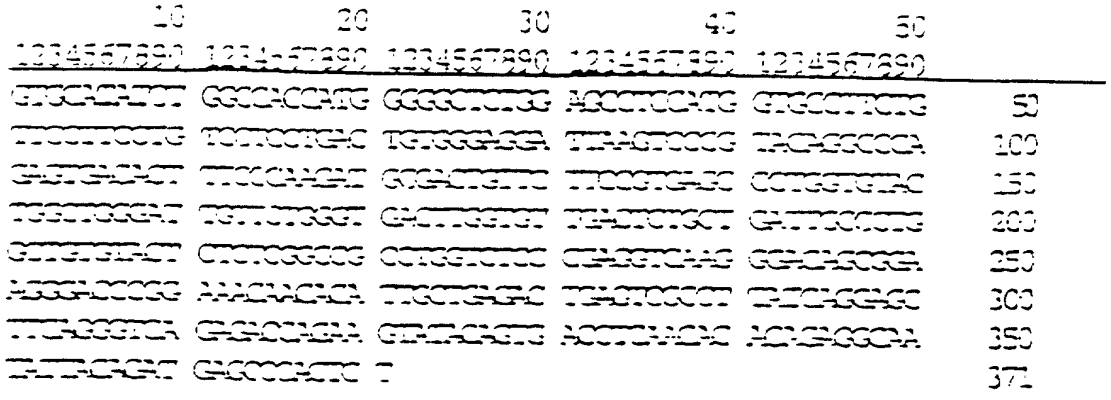

Figure 11B

SEQ ID n°12

Figure 12A

SEQ ID n°8

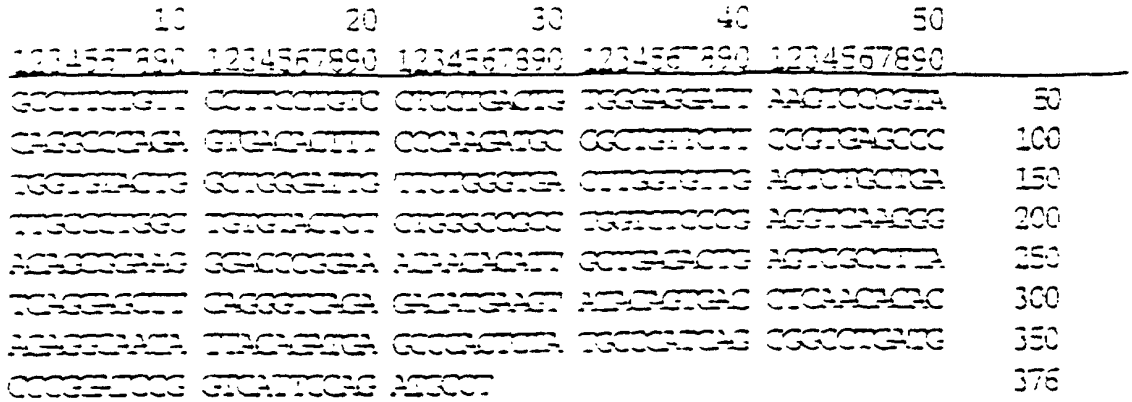

Figure 12B

SEQ ID n°13

Figure 13A

SEQ ID n°9

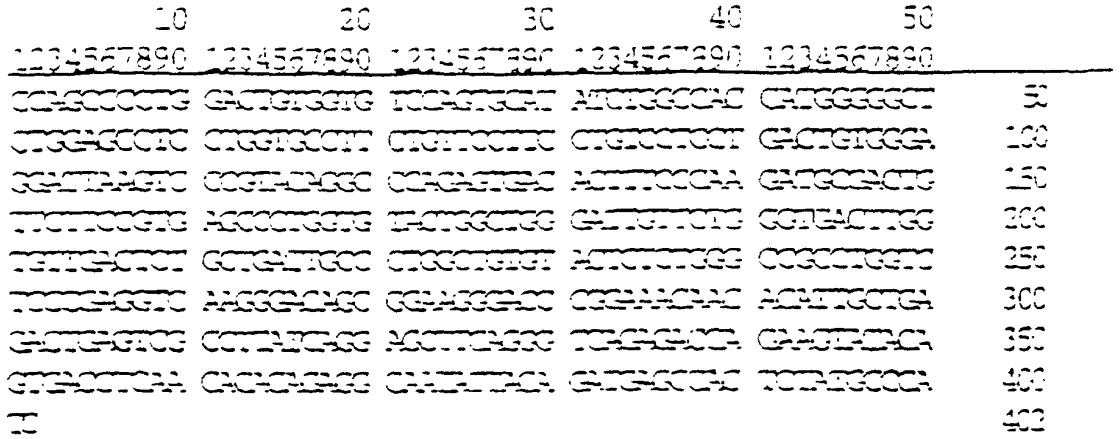

Figure 13B

SEQ ID n°14

Figure 14A

SEQ ID n°10

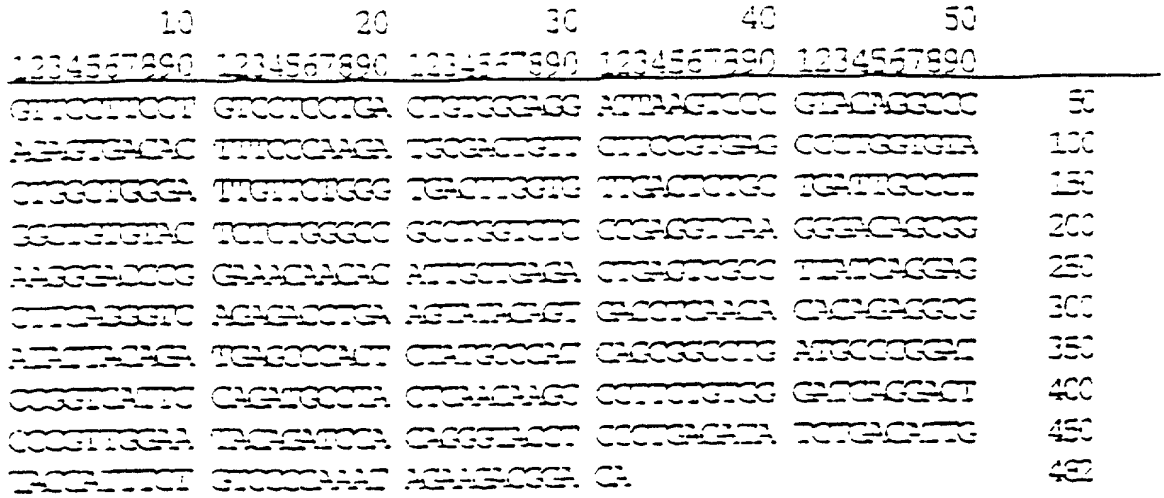

Figure 14B

SEQ ID n°15

Figure 15

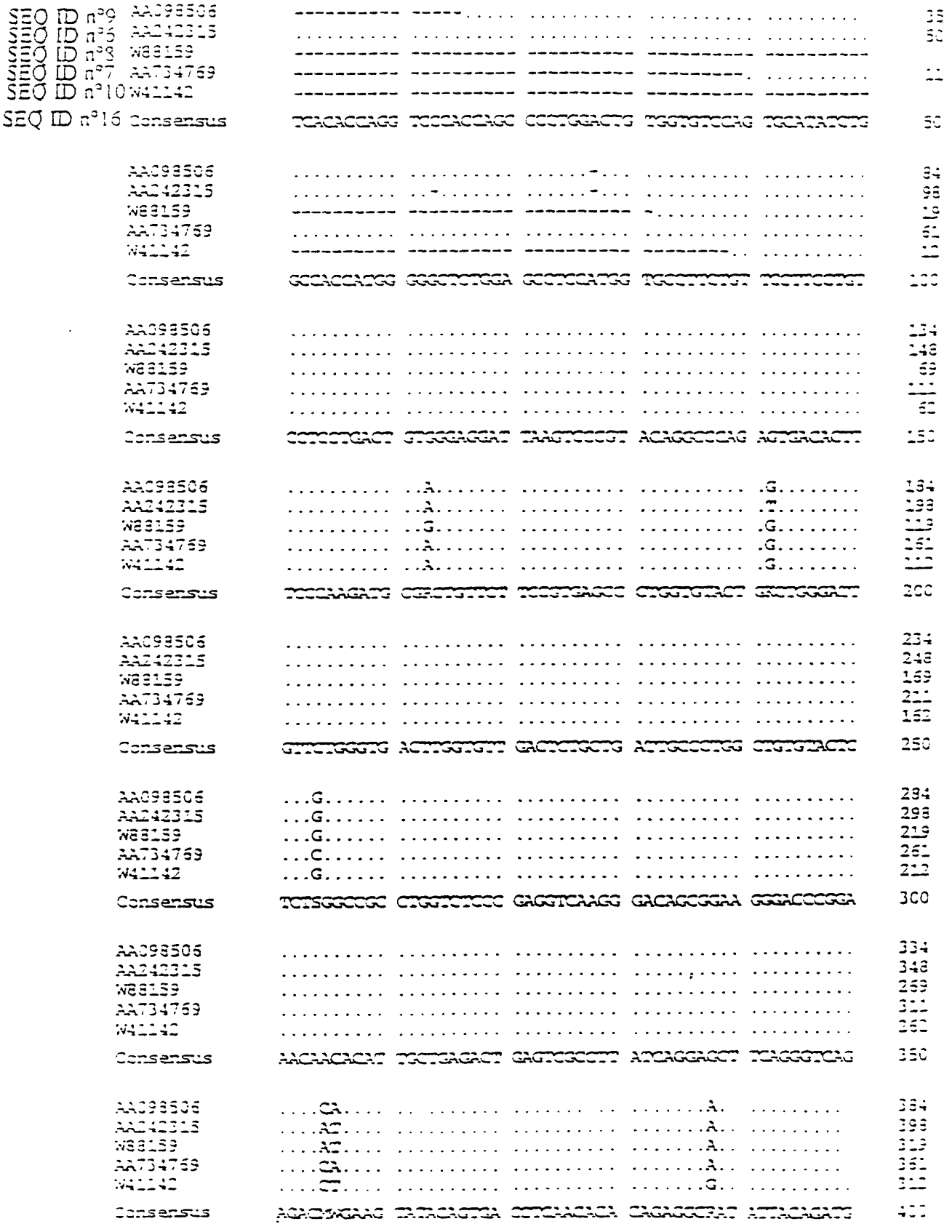
SEQ ID n°9 AA098506
SEQ ID n°6 AA242315
SEQ ID n°8 W88159
SEQ ID n°7 AA734769
SEQ ID n°10 W41142
SEQ ID n°16 Consensus
TCACACCAGG TCCCACCAGC CCCTGGACTG TGGTGTCCAG TGCATATCTG
GCCACCATGG GGGCTCTGGA GCCTCCATGG TGCCTTCTGT TCCTTCCTGT
CCTCCTGACT GTGGAGGAT TAAGTCCCGT ACAGGCCCAG AGTGACACTT
TCCCAAGATG CCRCTGTTCT TCCGTGAGCC CTGGTGTACT GRCTGGGATT
GTTCTGGGTG ACTTGGTGTT GACTCTGCTG ATTGCCCTGG CTGTGTACTC
TCTSGGCCGC CTGGTCTCCC GAGGTCAAGG GACAGCGGAA GGGACCCGGA
AACAACACAT TGCTGAGACT GAGTCGCCTT ATCAGGAGCT TCAGGGTCAG
AGACCWGAAG TATACAGTGA CCTCAACACA CAGAGCCTAT ATTACAGATC

Figure 15 (contd.)

```
AA098506    ....-..... . ... ...- ---------- ---------- ----------  402
AA242315    ..... ... . . ..... .......... ..... .... ..........  448
W88159      .......... .......... .......... ..... .... ..........  369
AA734769    .......... ---------- ---------- ---------- ----------  371
W41142      .......... .......... .......... .......... ..........  362

Consensus   AGCCCACTCT ATGCCCATCA GCGGCCTGAT GCCCGGATCC GGTCATTCCA  450

AA098506    ---------- ---------- ---------- ---------- ----------  402
AA242315    .......... .......... .-..C..A.. .......... ..........  497
W88159      .........--- ---------- ---------- ---------- ----------  376
AA734769    ---------- ---------- ---------- ---------- ----------  371
W41142      .......... .......... ....G..G.. .......... ..........  412

Consensus   GATGCCTACT CAACAAGCCC TTCTSTGRGA TCAGGACTCC CGTTGGAATA  500

AA098506    ---------- ---------- ---------- ---------- ----------  402
AA242315    .......... .........-- ---------- ---------- ----------  515
W88159      ---------- ---------- ---------- ---------- ----------  376
AA734769    ---------- ---------- ---------- ---------- ----------  371
W41142      .......... .......... .......... .......... ..........  462

Consensus   CAGATCCACA GGGTACCTCC CTGAGATATC TGACATTGTA CCATTTCTGT  550

AA098506    ---------- ----------                                   402
AA242315    ---------- ----------                                   515
W88159      ---------- ----------                                   376
AA734769    ---------- ----------                                   371
W41142      .......... ..........                                   482

Consensus   CCCCAAATAG AAGACGGACA                                   570
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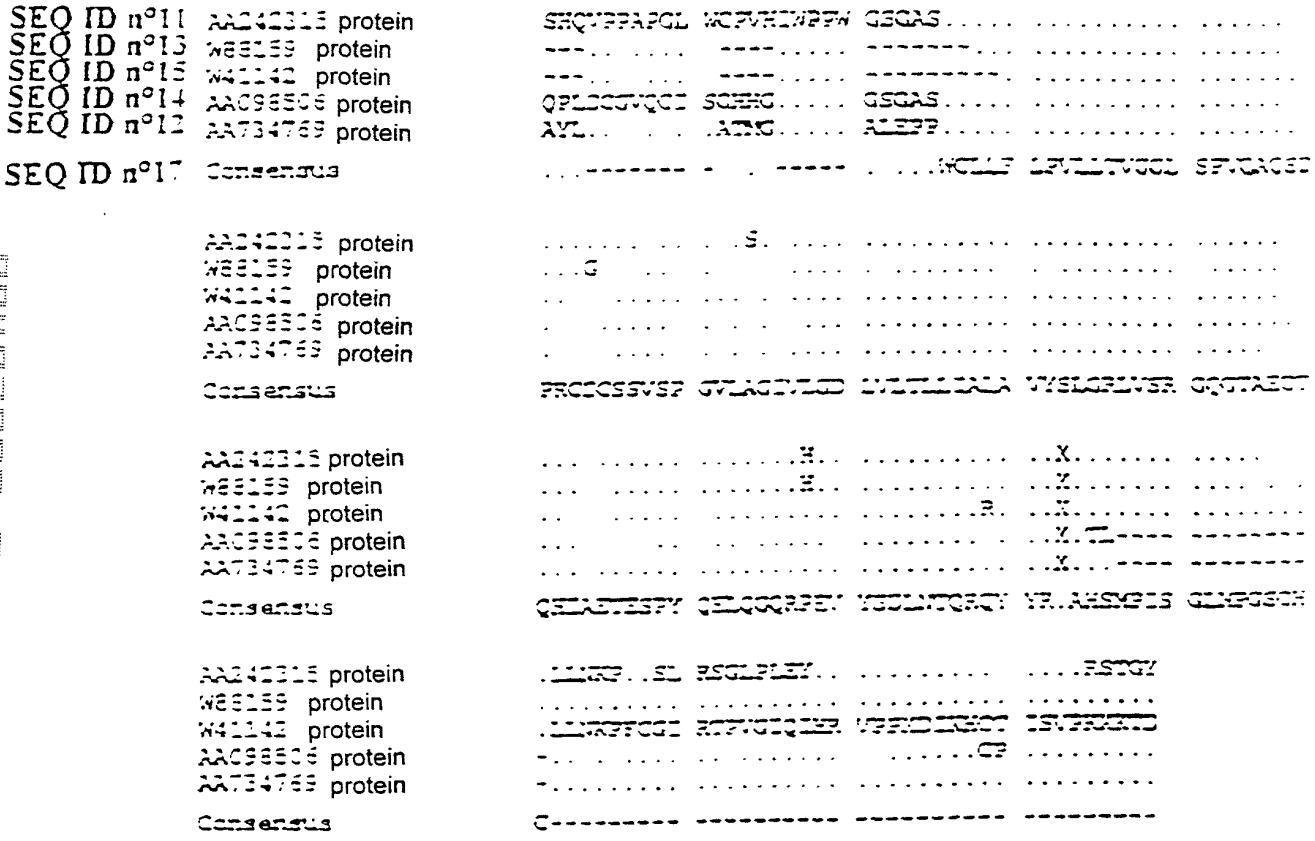
Figure 16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SEQ ID n°13 W88159 protein
SEQ ID n°15 W41142 protein
SEQ ID n°14 AAC98806 protein
SEQ ID n°12 AA734769 protein
SEQ ID n°17 Consensus
AAC42315 protein
W88159 protein
W41142 protein
AAC98806 protein
AA734769 protein
Consensus
AAC42315 protein
W88159 protein
W41142 protein
AAC98806 protein
AA734769 protein
Consensus
AAC42315 protein
W88159 protein
W41142 protein
AAC98806 protein
AA734769 protein
Consensus

Figure 1C

SEQ ID NO:1

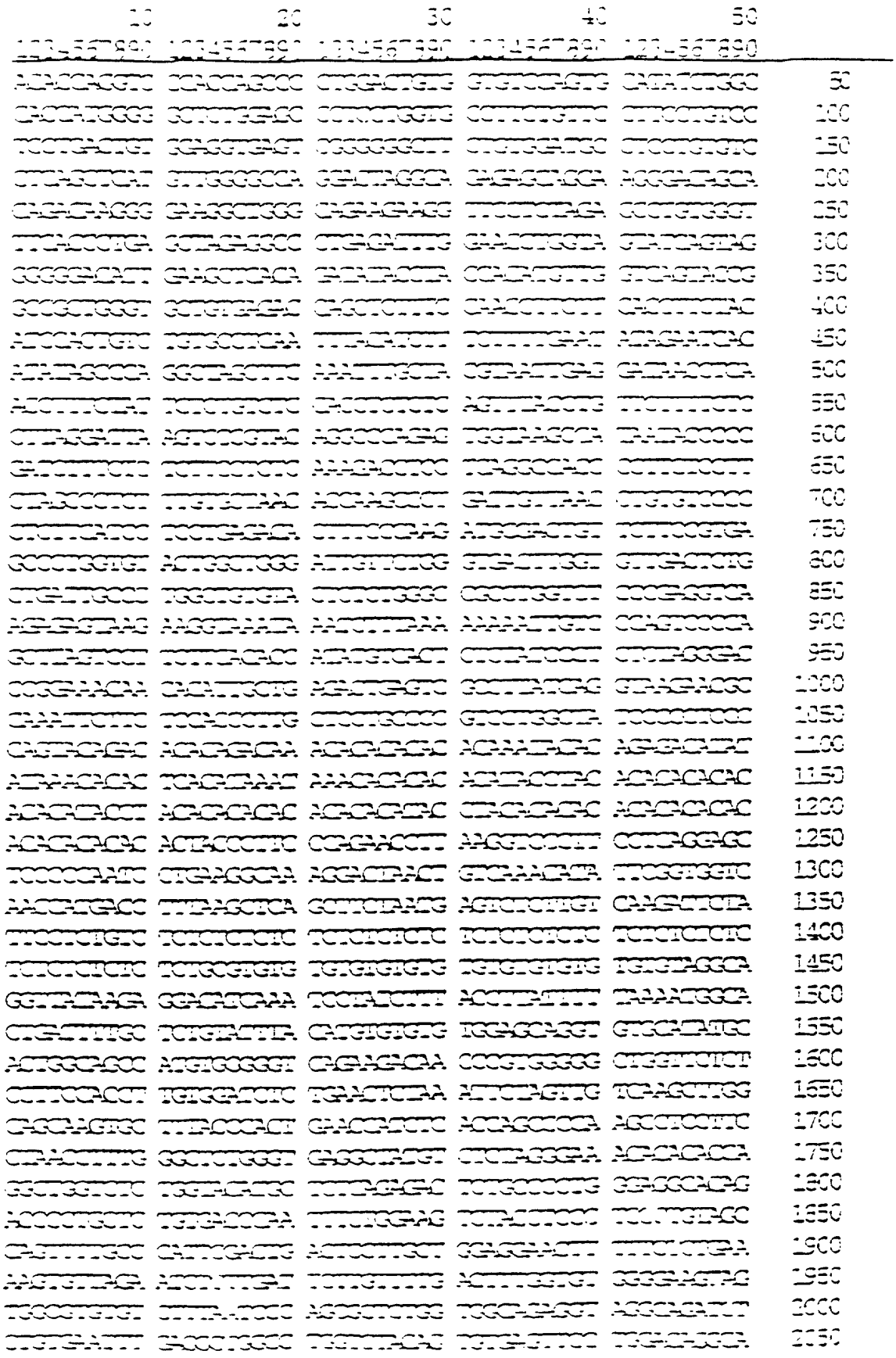

Figure 17 (contd.)

SEQ ID n° 18 (contd.)

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AACAAAACAA	AAAGAACCT	CAATATGGC	CACCTGTGT	CCAGAAGACC	2150
CCGGGCTGTC	TAGTTTTGA	GACCAGGAA	ACTTTAGGGC	AAATGTCAGC	2200
CTGATTTTTT	TACCCTTCGG	TATCTTGGTT	GAGGCTACA	TGGTCAACA	2250
CAGCACTCCA	ATTGGAGAG	CTTATTTGA	GCAACTTAC	AAAATCATTT	2300
GGGCTGACAT	TATGAGAGA	TTGAGTGA	CCAACATAT	GGTGGGACAG	2350
GAAGAAATT	GAGATGGGG	AAACTAAAC	ATGCCAACA	CTCAAGGGT	2400
GAGCAGTTG	AAGATCTGTG	CGCTTGGTGC	TCCAGGCATC	GGGGTGGGGG	2450
GCTGCACATG	TAAGAACCT	GGGGTTGGTG	CCTAATGTGC	AGGCAGAAG	2500
GCCAGGAGA	TGCTGAGTGC	ATTTGAATAA	AACCTTGACC	TTTCACGAC	2550
TTTAATTTG	AAAAACCTGC	CAGGACCT	GAAGTGAT	AGTAGCCTAG	2600
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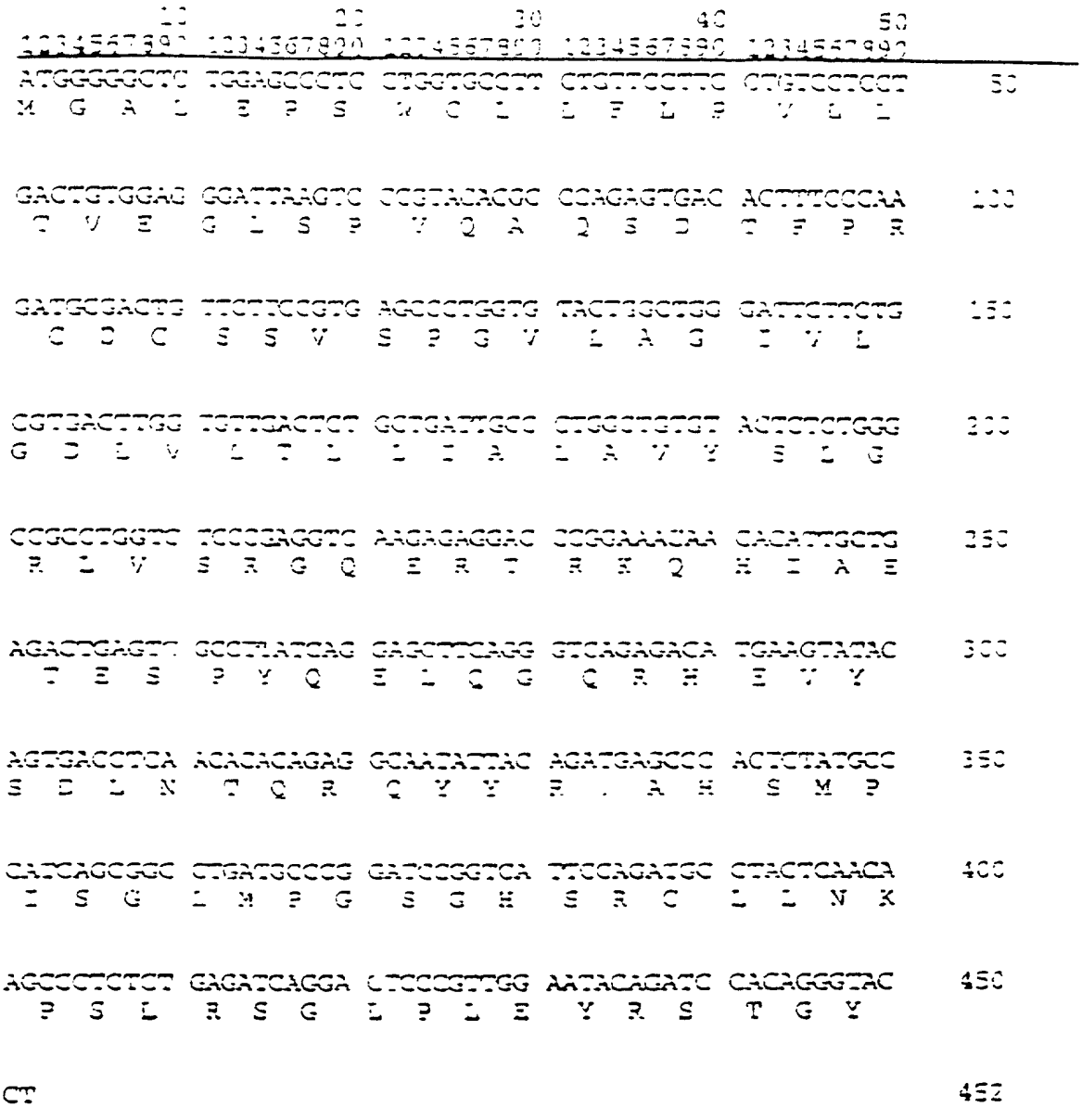
TTTAAGGAC	TTCTGGGTCA	GAGCTAGA	GTTACATTG	ACCTGAACAC	2700
ACAGAGGAA	TATTACAGAT	GAGCCATC	TATGCCATC	AGCCCCTGA	2750
TGCCTGAAT	CGGTGATCC	AGTGCTAC	TGAACAACC	CTCTCTGAA	2800
TCAGGATCC	CGTTGAATA	GAGTCCAA	GGGTACCT		2838

Figure 18

3' Intron sequence (donor site)	Exon sequence	5' Intron sequence (acceptor site)
	MetGly alGluG ATGGGG..-Exon 1-..TCCAGG	GAGGTGA....
....TCCTTAG	lyLeuS lnSerA GATTAA..-Exon 2-..AGAGTG	GTAAGCC...
....TCCTGAG	spThrP lnGluA ACACTT..-Exon 3-..AAGAGA	GTAAGAA..
....TCTCTAG	rgThrA TyrGln GGACCC..-Exon 4-..TATCAG	GTAAGAA....
....TTTTAAG		

Figure 19


10 20 30 40 50
1234567890 1234567890 1234567890 1234567890 1234567890
SEQ ID n°27 ATGGGGGCTC TGGAGCCCTC CTGGTGCCTT CTGTTCCTTC CTGTCCTCCT 50
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GACTGTGGAG GGATTAAGTC CCGTACACGC CCAGAGTGAC ACTTTCCCAA 100
T V E G L S P V Q A Q S D T F P R
GATGCGACTG TTCTTCCGTG AGCCCTGGTG TACTTGCTGG GATTCTTCTG 150
C D C S S V S P G V L A G I V L
CGTGACTTGG TGTTGACTCT GCTGATTGCC CTGGCTGTGT ACTCTCTGGG 200
G D L V L T L L I A L A V Y S L G
CCGCCTGGTC TCCCGAGGTC AAGAGAGGAC CCGCAAACAA CACATTGCTG 250
R L V S R G Q E R T R K Q H I A E
AGACTGAGTT GCCTTATCAG GAGCTTCAGG GTCAGAGACA TGAAGTATAC 300
T E S P Y Q E L Q G Q R H E Y Y
AGTGACCTCA ACACACAGAG GCAATATTAC AGATGAGCCC ACTCTATGCC 350
S D L N T Q R Q Y Y R . A H S M P
CATCAGCGGC CTGATGCCCG GATCCGGTCA TTCCAGATGC CTACTCAACA 400
I S G L M P G S G H S R C L L N K
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CT 452

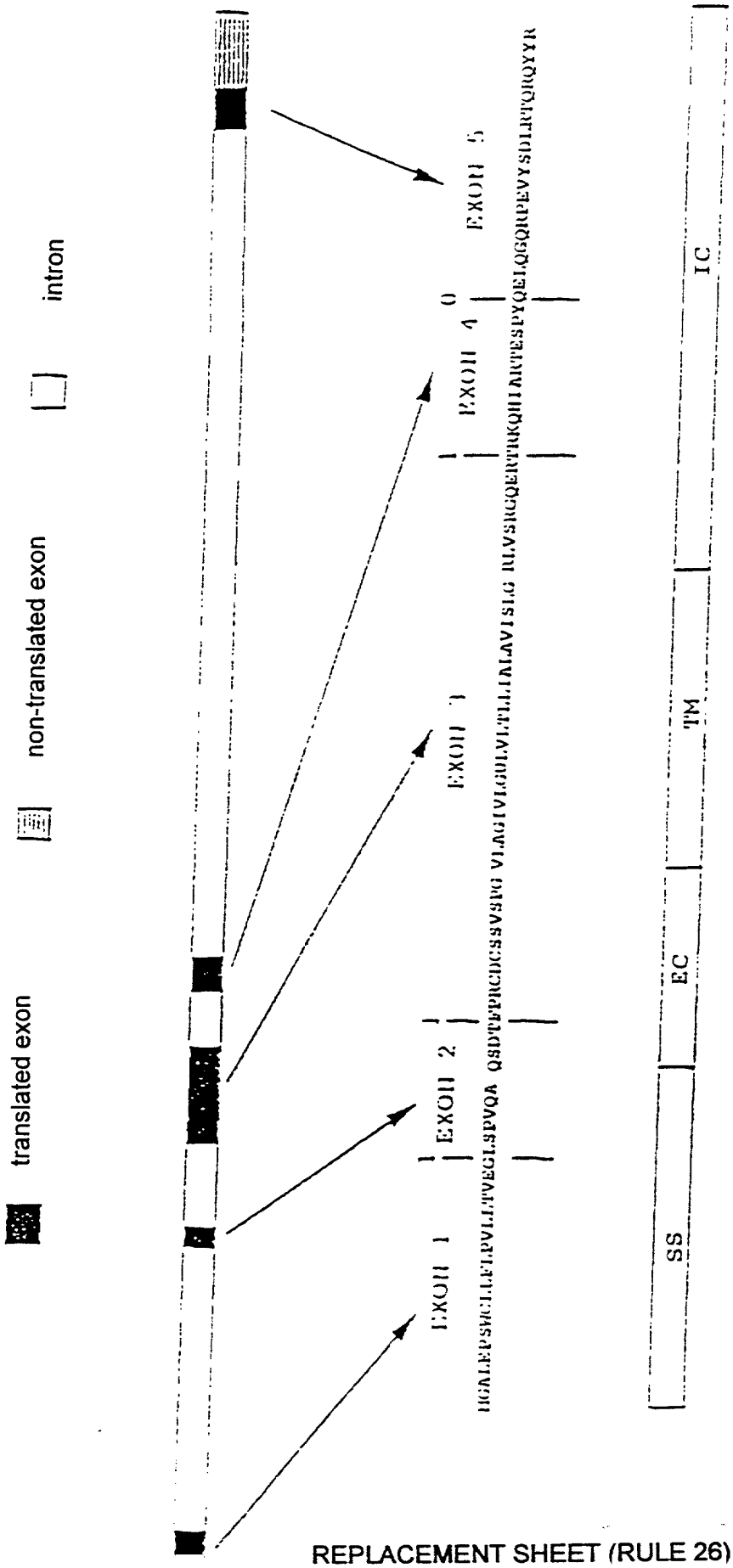

translated exon
non-translated exon
intron
EXON 1
EXON 2
EXON 3
EXON 4
EXON 5
SS
EC
TM
IC

Figure 20

Figure 21

SEQ ID n°31

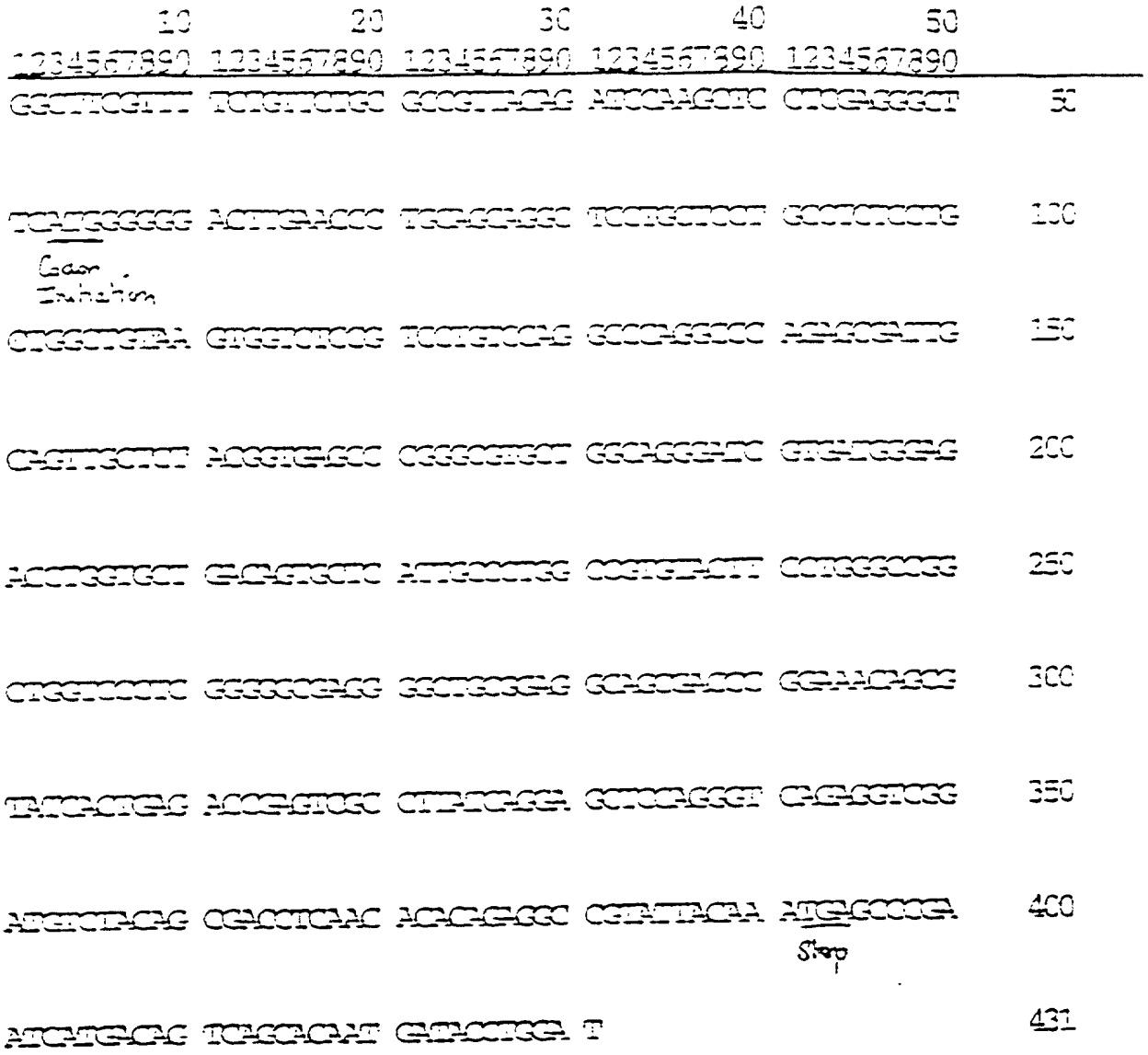


Figure 22

Release of serotonin induced by the p50/KARAP complex reconstituted in RBL-2H3 cells

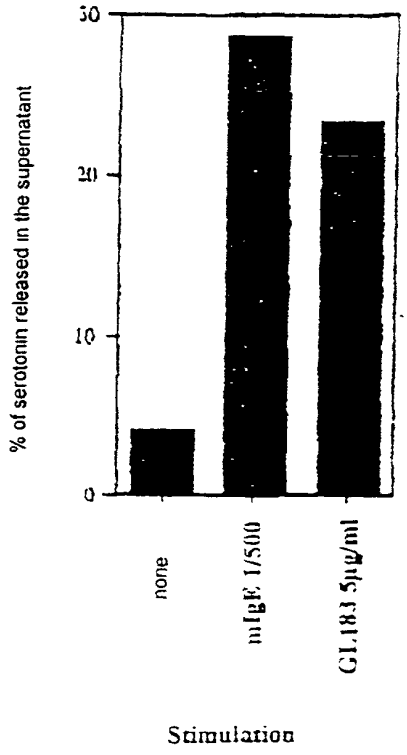
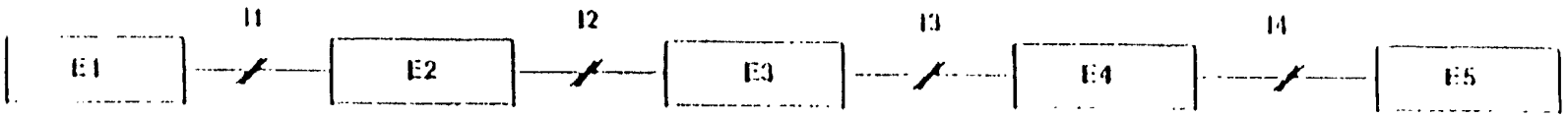


Figure 23



(bp)

> 62	404	30	148	134	180	46	2882	> 65	human KARAP
> 115	440	31	144	138	91	44	1666	> 102	murine KARAP

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Polypeptides associated with activator receptors and their biological applications

the specification of which (check applicable box(s)):

- [ ] is attached hereto.
- [ ] was filed on _____ as U. S. Application Serial No _____
- [X] was filed as PCT international application No. PCT/ FR98/00883 on April 30, 1998

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
97/05411	FR	30/04/97
98/00927	FR	28/01/98

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	Day/Month/Year Filed	Status: patented, pending, abandoned
PCT/FR98/00883	30/04/98	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352, Richard G. Besha, 22770, Mark E. Nusbaum, 32340; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Jerry D. Craig, 38026

(1) **Inventor's Signature** _____ Date April 30, 1998

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(2) **Inventor's Signature** _____ Date April 30, 1998

2-00 Inventor's Name (typed) Alessandro (First) _____ (Middle Initial) MORETTA (Family Name) IT (Citizenship)

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(3) **Inventor's Signature** _____ Date April 30, 1998

3-00 Inventor's Name (typed) Lucia (First) _____ (Middle Initial) OLCESE (Family Name) IT (Citizenship)

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FOR ADDITIONAL INVENTORS, check box [X] and attach sheet with same information and signature and date for each.

Inventor's Signature _____ Date April 30, 1998

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Residence (City) CASSIS (State/Foreign Country) FRANCE

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Inventor's Signature Elena Tomasello Date April 30, 1998

Inventor's Name (typed) Elena (First) _____ (Middle Initial) TOMASELLO (Family Name) FR (Citizenship)

Residence (City) MARSEILLE (State/Foreign Country) FRANCE

Post Office Address 38 allée des Pins Zip Code 13009

Inventor's Signature _____ Date _____

Inventor's Name (typed) _____ (First) _____ (Middle Initial) _____ (Family Name) _____ (Citizenship)

Residence (City) _____ (State/Foreign Country) _____

Post Office Address _____ Zip Code _____

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.

SEQUENCE LISTING

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Leu Gln Gly Gln Arg His Glu Val Tyr Ser Asp Leu Asn Thr Gln Arg
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Gln Tyr Tyr Arg Xaa Ala His Ser Met Pro Ile Ser Gly Leu Met Pro
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Gln Ser Asp Thr Phe Pro Arg Cys Asp Cys Ser Ser Val Ser Pro Gly
         35                  40                  45

Val Leu Ala Gly Ile Val Leu Gly Asp Leu Val Leu Thr Leu Leu Ile
     50                  55                  60

Ala Leu Ala Val Tyr Ser Leu Gly Arg Leu Val Ser Arg Gly Gln Gly
 65                  70                  75                  80

Thr Ala Glu Gly Thr Arg Lys Gln His Ile Ala Glu Thr Glu Ser Pro
                 85                  90                  95

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Leu Ile Ala Leu Ala Val Tyr Ser Leu Gly Arg Leu Val Ser Arg Gly
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Gln Gly Thr Ala Glu Gly Thr Arg Lys Gln His Ile Ala Glu Thr Glu
 65                  70                  75                  80

Ser Pro Tyr Gln Glu Leu Gln Gly Gln Arg His Glu Val Tyr Ser Asp
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Leu Asn Thr Gln Arg Gln Tyr Tyr Arg Xaa Ala His Ser Met Pro Ile
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Gly Gly Leu Ser Pro Val Gln Ala Gln Ser Asp Thr Phe Pro Arg Cys
         35                  40                  45

Asp Cys Ser Ser Val Ser Pro Gly Val Leu Ala Gly Ile Val Leu Gly
     50                  55                  60

Asp Leu Val Leu Thr Leu Leu Ile Ala Leu Ala Val Tyr Ser Leu Gly
 65                  70                  75                  80

Arg Leu Val Ser Arg Gly Gln Gly Thr Ala Glu Gly Thr Arg Lys Gln
                 85                  90                  95

His Ile Ala Glu Thr Glu Ser Pro Tyr Gln Glu Leu Gln Gly Gln Arg
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Val Leu Ala Gly Ile Val Leu Gly Asp Leu Val Leu Thr Leu Leu Ile
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     50                  55                  60

Thr Ala Glu Gly Thr Arg Lys Gln His Ile Ala Glu Thr Glu Ser Pro
 65                  70                  75                  80

Tyr Gln Glu Leu Gln Gly Gln Arg Pro Glu Val Tyr Ser Asp Leu Asn
                 85                  90                  95

Thr Gln Arg Arg Tyr Tyr Arg Xaa Ala His Ser Met Pro Ile Ser Gly
            100                 105                 110

Leu Met Pro Gly Ser Gly His Ser Arg Cys Leu Leu Asn Lys Pro Phe
        115                 120                 125

Cys Gly Ile Arg Thr Pro Val Gly Ile Gln Ile His Arg Val Pro Pro
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Val Ser Pro Gly Val Leu Ala Gly Ile Val Leu Gly Asp Leu Val Leu
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Thr Leu Leu Ile Ala Leu Ala Val Tyr Ser Leu Gly Arg Leu Val Ser
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Arg Gly Gln Gly Thr Ala Glu Gly Thr Arg Lys Gln His Ile Ala Glu
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Thr Glu Ser Pro Tyr Gln Glu Leu Gln Gly Gln Arg Pro Glu Val Tyr
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35 40 45

Val Leu Gly Asp Leu Val Leu Thr Leu Leu Ile Ala Leu Ala Val Tyr
50 55 60

Ser Leu Gly Arg Leu Val Ser Arg Gly Gln Glu Arg Thr Arg Lys Gln
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cgacctcaac acacagaggc cgtattacaa atgagcccga atcatgacag tcagcacaat 420
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* * * * * * * * * * *

October 29, 1999

Assistant Commissioner for Patents
Washington, DC 20231

REQUEST TO THE CHIEF DRAFTSPERSON

Sir:

Authorization is requested to amend Figure 16 to add the material underlined in red in the attached new Figure 16. No new matter has been added by this amendment for the reasons noted in the attached Preliminary Amendment.

Respectfully submitted,

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By: _____

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New Figure 16
(aa consensus seq)

FIGURE 16

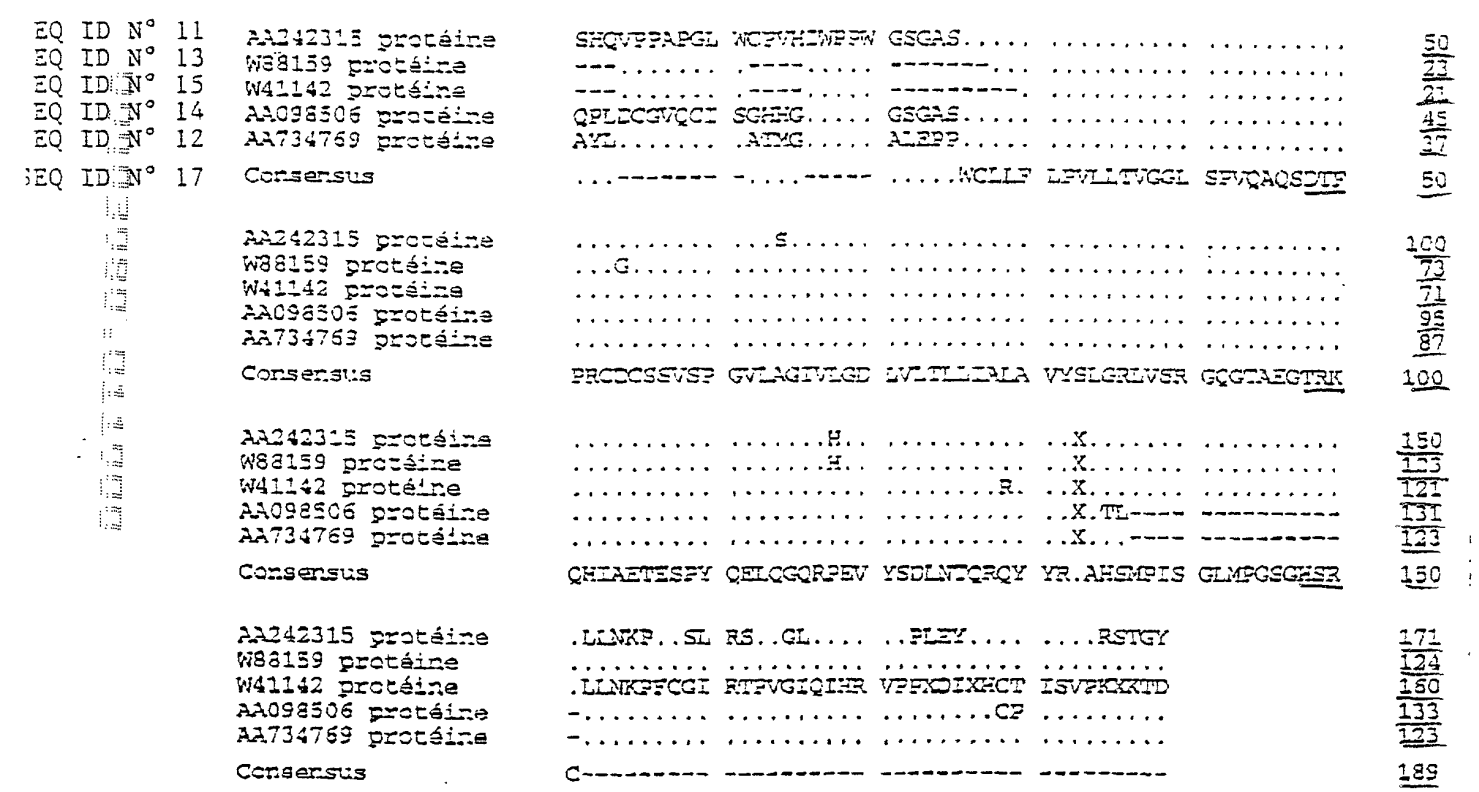
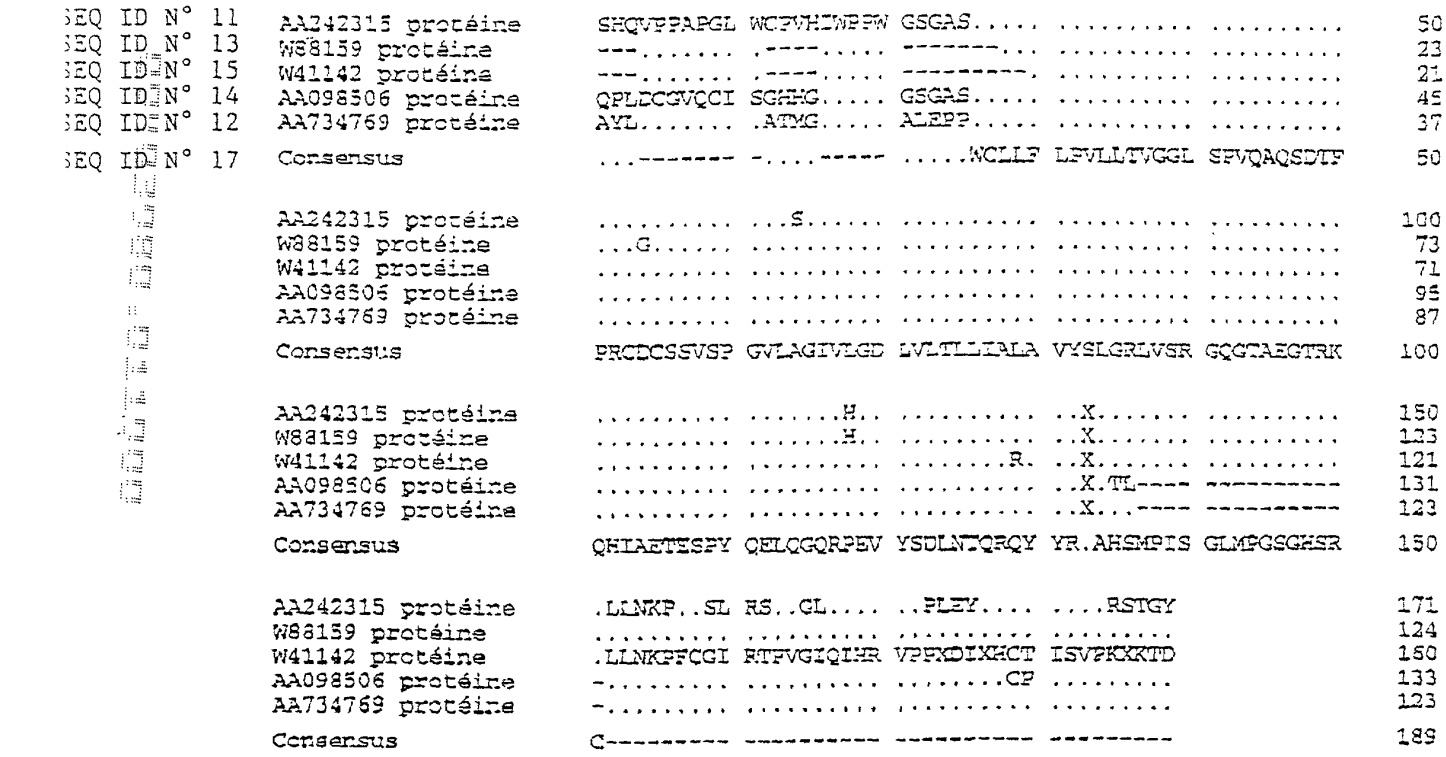


FIGURE 16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SEQ ID N° 13 W88159 protéine ---..... .----..... -------... 23
SEQ ID N° 15 W41142 protéine ---..... .----..... ----------. 21
SEQ ID N° 14 AA098506 protéine QPLECGVQCI SGHHG..... GSGAS..... 45
SEQ ID N° 12 AA734769 protéine AYL..... .ATMG..... ALEPP..... 37
SEQ ID N° 17 Consensus ...-------- -....----- .....WCLLF LFVLLTVGGL SFVQAQSDTF 50
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W41142 protéine 71
AA098506 protéine 95
AA734769 protéine 87
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W41142 protéine ....R. ..X..... 121
AA098506 protéine ....X.TL---- ---------- 131
AA734769 protéine ....X...---- ---------- 123
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W88159 protéine 124
W41142 protéine .LLNKPFCGI RTPVGIQIHR VPPKDIXHCT ISVPKXKTD 160
AA098506 protéine -..... ....CP 133
AA734769 protéine -..... 123
Consensus C---------- ---------- ---------- --------- 189